Remarks

Status of Claims

Claims 13, 20 and 30 are canceled herein without prejudice or disclaimer. Applicants reserve the right to pursue subject matter encompassed by all canceled claims in one or more divisional or continuation applications. Claims 11 and 25 have been amended to further define the claimed embodiments as discussed below. Support for the amended claims 11 and 25 can be found, for example, in Table 1D (angiogenesis) and page 1538, paragraphs [0667] and [0668] of the substitute specification (contiguous). Claims 11, 12, 16, 21, 24-29, 31 and 32 will be pending upon entry of this amendment. The Examiner has withdrawn claims 21, 31 and 32 from consideration "as being drawn to a non-elected invention." Applicants thank the Examiner for acknowledging the request for rejoinder of the withdrawn method claims upon allowance of the corresponding product claims.

I. Objection to the Specification

The Examiner has objected to the specification because a) not all of the trademarks disclosed are capitalized and accompanied by the generic terminology; and b) it contains embedded hyperlinks and/or other form of browser-executable code. Applicants have amended the specification in the substitute specification provided herewith to address the Examiner's objections. Accordingly, Applicants respectfully submit that this objection has been obviated and should be withdrawn.

II. Sequence Compliance

The Examiner has noted that the sequence listing statement submitted by Applicants in response to the Notice of Missing Parts does not indicate that there is no new matter. Applicants contend that the requirement to state that there is no new matter is only "where applicable." Accordingly, Applicants resubmission of the original sequence listing and statement filed on September 20, 2003 to replace the lost sequence listing and statement under 37 C.F.R. § 1.821(f) should not require a statement that there is no new matter. However, solely to advance prosecution of this application, Applicants attach herewith a statement that there is no new matter.

The Examiner has additionally noted that Applicants amended Table 1A in the previous response and has urged Applicants file a new sequence listing if this amendment has affected the previously filed sequence listing. Applicants submit that the amended Table 1A has not affected the previously filed sequence listing and therefore, a new sequence listing is not necessary.

III. Rejections Under 35 U.S.C. § 101

The Examiner has rejected claims 11-13, 16 and 25-29 under 35 U.S.C. § 101 as allegedly lacking a credible, substantial, specific, or well-established utility. In particular, the Examiner has alleged that "[t]he specification fails to provide objective evidence of any activity for the claimed proteins." *See*, Paper No. 20061028, pages 4-8.

Applicants respectfully disagree and traverse. The specification as filed provides many biological functions associated with the claimed polypeptides in Table 1D. *See*, for example, pages 296-309 of the substitute specification. These functions were identified via high-throughput screening of the polypeptides as described in Examples 22 and 24-27. For example, SEQ ID NO:408 was demonstrated to induce production of ICAM and VCAM in endothelial cells. It was well known in the art at the time of filing that ICAM and VCAM were able to promote angiogenesis and were implicated in tumor neovascularization. *See*, for example, Regidor et al. 1998, Koch et al. 1995 and Fox et al. 1995, submitted herewith as Exhibits A-C, respectively. Based on this information, Applicants listed the highly preferred indications for HACCI17 polypeptides in the last column of Table 1D of the specification as including "neoplastic disorders (e.g. cancer/tumorigenesis)." Furthermore, Applicants listed the examples of "leukemia, lymphoma, melanoma, renal cell carcinoma, and prostate, breast, lung, colon, pancreatic, esophageal, stomach, brain, liver and urinary cancer." The usefulness of targeting HACCI17 polypeptides as a potential antiangiogenic cancer therapy is further substantiated by a recent publication.[1] Hewitt et al. (2006), submitted herewith as Exbibit D, demonstrates that CLDN5 (also known as claudin 5) is highly expressed in

[1] Applicants point out that post-filing date scientific papers, such as the paper discussed herein, may be used to corroborate Applicants' asserted utility. Legal precedent for the use of post-filing date references in this manner can be found in In re Brana, where the Federal Circuit stated that:

> The Kluge declaration, though dated after applicants' filing date, can be used to substantiate any doubts as to the asserted utility since this pertains to the accuracy of a statement already in the specification. In re Marzocchi, 439 F.2d at 224 n.4, 169 U.S.P.Q. (BNA) at 370 n.4. 51 F.3d 1560, 1567, 34 U.S.P.Q.2D (BNA) 1436 (Fed. Cir. 1995).

vascular endothelial cells but generally not expressed in epithelial tissue and the authors conclude that it is a possible target for antiangiogenic therapy. Applicants submit that CLDN5 (claudin 5) is 100% identical to SEQ ID NO:408 and provide a sequence alignment herewith as Exhibit E.

Contrary to the Examiner's allegation, the instant case is not analogous to the situation in *Brenner v. Manson* (383 U.S. 519 (U.S. 1966)). In *Brenner*, the issue was not whether a disclosed utility was sufficient. Rather, the applicant was trying to establish an earlier date of invention for the purpose of provoking an interference (*Id.* at 521). Indeed, in *Brenner* the Examiner's initial basis for refusing to declare an interference was that the applicant had failed to disclose any utility at all (*Id.* at 521). Thus, the issue in *Brenner* was whether the applicant had made an adequate "showing" to establish a prior date of invention, *i.e.*, whether "the process claim has been reduced to production of a product shown to be useful" through actual demonstration of the utility (*Id.* at 534). The only evidence offered by the applicant to make this showing was a reference to an article by a third party showing the activity of an adjacent homologue of the subject steroid compound (*Id.* at 521-522). The appellate court agreed that the applicant had done nothing to show or demonstrate that the compound was indeed useful (*Id.* at 521). Thus, it upheld the rejection of the request for declaration of an interference (*Id.* at 536).

In contrast, the issue in the present case is whether the instant application explicitly teaches a utility that meets the requirements of 35 U.S.C. § 101. Applicants point out that the specification need only make *one* credible assertion of utility for the claimed invention to satisfy 35 U.S.C. § 101. *See, e.g., Raytheon v. Roper*, 724 F.2d 951, 958, 220 U.S.P.Q. 592, 598 (Fed. Cir. (1983), *cert. denied*, 469 U.S. 835 (1984). The disclosure of the use of HACCI17 polypeptides for a number of specific disorders does not negate the specificity of any one of those uses. Indeed, the M.P.E.P. at § 2107.02 states "[i]t is common and sensible for an applicant to identify several specific utilities for an invention . . .". Further, "[i]f applicant makes one credible assertion of utility, utility for the claimed invention as a whole is established." *Id. See also, In re Malachowski*, 189 U.S.P.Q. 432 (C.C.P.A. 1976); *Hoffman v. Klaus*, 9 U.S.P.Q.2d 1657 (Bd. Pat. App. & Inter. 1988).

Applicants note that the test for specificity is whether an asserted utility is specific to the subject matter claimed, in contrast to a utility that would be applicable to the broad class of the invention. *See* M.P.E.P § 2107.01 on page 2100-32. Accordingly, the disclosed utility

for the HACCI17 polypeptides discussed above is specific, in that not every polypeptide is useful for the diagnosis and/or treatment of the above-mentioned disorders.

Moreover, where the specification discloses a biological activity (e.g., production of ICAM and VCAM), and reasonably correlates that activity to a disease condition (e.g., neoplastic diseases such as cancer/tumorigenesis), the specification has sufficiently identified a specific utility for the invention. M.P.E.P. § 2107.01 at 2100-32 (emphasis added). Applicants point out that the specification does not have to prove that a correlation exists between a particular activity and an asserted therapeutic use of a compound as a matter of statistical certainty or provide actual evidence of success in treating humans where such a utility is asserted. See M.P.E.P. § 2107.01(III). All that is required of Applicants is that there be a reasonable correlation between the biological activity and the asserted utility (*See,* Nelson v. Bowler, 626 F.2d at 857). *See also*, Fujikawa v. Wattanasin, 39 U.S.P.Q.2d 1895 (Fed. Cir. 1996). Applicants submit that, based on the present specification, the ordinary skilled artisan would readily recognize the specific asserted utility of the claimed polypeptides.

Applicants respectfully remind the Examiner that utility can exist for therapeutic inventions "despite the fact that an applicant is at a very early stage in the development of a pharmaceutical product or therapeutic regimen based on a claimed pharmacological or bioactive compound or composition." M.P.E.P. § 2107(III) at 2100-35. "Usefulness in patent law . . . necessarily includes the expectation of further research and development. The stage at which an invention in this field becomes useful is well before it is ready to be administered to humans." *In re* Brana, 51 F.3d 1560, 1568 (Fed. Cir. 1995) (Emphasis added).

Furthermore, the Examiner alleges that the claimed invention is not supported by a substantial utility. As discussed above, Applicants assert that based on what is disclosed in the specification, coupled with what was known in the art on the earliest effective priority date of the present invention, it is reasonable that the claimed invention is useful in the diagnosis and/or treatment of certain disorders, and that such uses fulfill an unmet medical need. The M.P.E.P. states, "any reasonable use that an applicant has identified for the invention that can be viewed as providing a public benefit should be accepted as sufficient, at least with regard to defining a 'substantial' utility." *See* M.P.E.P. § 2107.01(I). Applicants thus assert that the claimed invention is supported by a substantial or "real world" utility. Accordingly, Applicants submit that the specification as filed provides at least one specific,

substantial and credible utility for the claimed polypeptides and respectfully request that this rejection be reconsidered and withdrawn.

The Examiner has additionally rejected claims 11-12 and 25-28 under 35 U.S.C. § 101 as allegedly drawn to non-statutory subject matter (a protein). Applicants have amended claims 11 and 25 herein to obviate this rejection. Accordingly, Applicants respectfully request that this rejection be reconsidered and withdrawn.

IV. Rejections Under 35 U.S.C. § 112, first paragraph, Enablement

A. Alleged Lack of Utility

The Examiner has rejected claims 11-13, 16 and 25-29 under 35 U.S.C. § 112, first paragraph, alleging that since the claimed invention is not supported by either a specific and substantial or a well-established utility, one skilled in the art would not know how to use the claimed invention. *See*, Paper No. 20061028, page 8. In view of the arguments presented above in response to the rejection under 35 USC § 101, Applicants submit that the claims are supported by a specific, substantial, and credible asserted utility, and thus adequately teach how to use the invention. Accordingly, it is requested that the instant rejection be reconsidered and withdrawn.

B. Fragments and 95% Identical Variants

The Examiner has rejected claims 11-13, 16 and 25-29 under 35 U.S.C. § 112, first paragraph. In particular, the Examiner alleged "the amount of experimentation required to practice the claimed invention is undue as the claims encompass an unspecified amount of fragments that are not supported by the instant specification." *See*, Paper No. 20061028, pages 8-11. The Examiner further alleged:

> The claimed polypeptide once modified might not have the same properties of the native/wild-type protein or retain the same function. The claims recite language such as "at least 30 amino acids...wherein said fragment has biological activity", however, no specific activity is disclosed. Note also that the 30 amino acid residues does not have to be contiguous and the claims do not indicate where variations will occur or what variations can be tolerated in the sequence.

See, Paper No. 10252005, pages 3-4, item 8.

Applicants respectfully disagree and traverse.

As an initial matter, Applicants have amended claims 11 and 25 herein to recite fragments as comprising at least 30 or 100 contiguous amino acids and that have a specific biological activity (i.e. promoting angiogenesis). The following comments are directed to the enablement of the claims as amended.

To satisfy the enablement requirement, the specification must enable a person of ordinary skill in the art to practice a single use of the claimed polypeptides without undue experimentation. *See, e.g.,* MPEP §2164.01(c). To make a proper enablement rejection, the examiner has the initial burden to establish a reasonable basis to question the enablement provided for the claimed invention. MPEP §2164.04; *see also, In re Wright*, 999 F.2d 1557, 1561-1562, 27 USPQ2d 1510, 1513 (Fed. Cir. 1993). Applicants respectfully submit that the Examiner has not provided sufficient evidence or a basis to question the enablement provided in the specification for the claimed polypeptides.

The Federal Circuit has held that *making the claimed species and screening them for function is acceptable*, as long as the experimentation is not undue. As in all cases, this is the test: whether it would require undue experimentation to practice the invention – even when a claim might encompass some inoperative embodiments. *See generally, Atlas Powder v. E.I. Du Pont de Nemours & Co.* 750 F.2d 1569, 224 U.S.P.Q. (BNA) 409 (Fed. Cir. 1984). Therefore, it is clearly not *per se* undue to make and test several fragments and variants, particularly when specific guidance was clearly disclosed in the specification coupled with what was known in the art at the time the invention was filed.

At the time the invention was filed, one of skill in art could envision the claimed variants and fragments. Contrary to the Examiner's contention that an infinite number of variants and fragments have been claimed, Applicants submit that the pending claims as amended herein are directed to variants that are 95% identical to SEQ ID NO:408 and/or comprise at least 30 or 100 contiguous amino acids of SEQ ID NO:408 that promote angiogenesis. This represents a finite number of species claimed. Methods of making these fragments and variants are described in detail in the substitute specification at pages 1431-1448. Additionally, these sections of the specification also teach how to identify preferred regions of the polypeptides and conservative amino acid substitutions.

Furthermore, it was *routine* at the time the specification was filed to determine empirically that particular fragments and variants of HACCI17 protein have the biological activity of the native protein. Biological functions associated with SEQ ID NO:408 are

provided in Table 1D of the specification as discussed in section III of this response. Additionally, Table 1D teaches exemplary assays that can be used to test for these functions. Moreover, it was well known in the art at the time of filing how to screen polypeptides for angiogenic function. Hence, the specification as filed provides ample guidance for making and screening the claimed variants and fragments and therefore, any experimentation required to practice the claims would not be undue. Accordingly, Applicants respectfully request this rejection be reconsidered and withdrawn.

V. Rejections Under 35 U.S.C. § 112, first paragraph, Written Description

A. Fragments and Variants

The Examiner has rejected claims 11-13, 16 and 25-29 under 35 U.S.C. 35 § 112, first paragraph, as allegedly containing subject matter which was not described in the specification in such a way as to reasonably convey to one skilled in the relevant art that the inventor(s), at the time the application was filed, had possession of the claimed invention. Specifically, the Examiner alleges that the claimed invention encompasses a genus of fragments of SEQ ID NO: 408, however, "no function is associated with the protein *per se*." To support of the above-cited rejection, the Examiner has referenced Vas-Cath Inc. v. Mahurkar, 935 F.2d 1555 (Fed. Cir. 1991) and Fiers v. Revel, 25 USPQ2d 1601 at 1606 (CAFC 1993). *See*, Paper No. 20061028, pages 11-14.

Applicants respectfully disagree. As an initial matter, Applicants submit that the specification as filed listed many biological functions associated with the claimed polypeptides in Table 1D as discussed in detail in section III. Therefore, Applicants respectfully disagree that "no function is associated with the protein *per se*." Applicants submit that claim 13 has been canceled herein, rendering the rejection as it pertains to this claim moot. With regards to the remaining claims, Applicants submit the following remarks.

The test for the written description requirement is whether one skilled in the art could reasonably conclude that the inventor has possession of the claimed invention in the specification as filed. (*See*, M.P.E.P. § 2163(I), and *Vas-Cath Inc. v. Mahurkar*, 935 F.2d 1555, 1563, 19 USPQ2d 1111, 1116 (Fed. Cir. 1991)).

The Federal Circuit has re-emphasized the well-settled principle of law that "[t]he written description requirement does not require the applicant 'to describe exactly the subject matter claimed, [instead] the description must clearly allow persons of ordinary skill in the art

to recognize that [he or she] invented what is claimed,'" *Union Oil Company of California v. Atlantic Richfield Company*, 208 F.3d 989, 54 U.S.PQ.2d 1227 (Fed. Cir. 2000). Further, the Federal Circuit has emphasized the importance of what the person of ordinary skill in the art would understand from reading the specification; and not whether the specific embodiments had been explicitly described or exemplified. Indeed, the court noted that "the issue is whether one of skill in the art could derive the claimed ranges from the patent's disclosure." *Union Oil Company of California v. Atlantic Richfield Company*, 208 F.3d at 1001, (emphasis added).

The Examiner has cited Fiddes v. Baird to support the argument that the rejected claims are unpatentable due to lack of written description. Applicants respectfully submit that the situation in Fiddes v. Baird is not applicable to the instant application. In Fiddes v. Bairds, the disputed '455 patent contained no disclosure of any polynucleotides of the claims, only a theoretical DNA sequence that was later proven incorrect. However, in the instant application, Applicants disclose the sequence of the claimed polypeptides (SEQ ID NO:408). Additionally, the specification provides the parameters used to determine the percent identity of the claimed polypeptides of the invention. *See*, for example, pages 1435-1436 of the substitute specification. Furthermore, Applicants teach that these variants often retain the biological activity of the original polypeptide and further teach conservative amino acid substitutions and other alterations which are more likely to conserve the biological activity of the polypeptide. *See*, for example, pages 1437-1440 of the substitute specification. Fragments of the polypeptide are similarly discussed in the substitute specification on pages 1444-1448. As noted above, Table 1D teaches the functions of HACCI17 (SEQ ID NO:408) polypeptides and provides representative assays to test for these functions. Therefore, Applicants submit that the specification describes with reasonable clarity to one of skill in the art that the inventors were in possession of the claimed invention on the earliest filing date of the present application. Applicants further submit that the Examiner has underestimated both the teaching of the present application and the level of skill in the art on the priority date of the present application.

Accordingly, from reading the specification, the skilled person would immediately recognize that, at the time the specification was filed, the Applicants had "invented what is claimed" (*Vas-Cath*, 935 F.2d at 1563); namely, a genus of polypeptides of SEQ ID NO:408, including fragments that promote angiogenesis. Therefore, the specification contains an adequate written description of the claimed polypeptides.

The Examiner has also referenced Vas-Cath Inc. v. Mahurkar, 935 F.2d 1555 (Fed. Cir. 1991) to allegedly support lack of written description of the pending claims. Applicants respectfully disagree and traverse the rejection on this basis. As an initial matter, the question in Vas-Cath Inc. v. Mahurkar, 935 F.2d 1555 (Fed. Cir. 1991) was whether or not the applicants earliest filed drawings (without any accompanying descriptive text) provided adequate written description under 35 U.S.C. § 112, first paragraph, for the claims in applicants later filed patent applications. In assessing the situation the Federal Circuit particularly noted "These [earlier cited] cases support our holding that, under proper circumstances, drawings alone may provide a 'written description' of an invention as required by § 112." Vas-Cath at 1565 (emphasis added). The Federal Circuit then reversed the previous district court's grant of summary judgment against Vas-Cath, Inc. and remanded the cases "for further proceedings consistent herewith." Vas-Cath at 1567.

In regard to the present application, Applicants disagree with the implication that the situation in Vas-Cath provides grounds for rejecting the currently pending claims as lacking adequate written description. Indeed, as detailed above, it is respectfully submitted that the present application provides more than sufficient written description to clearly allow persons of ordinary skill in the art to recognize that applicants invented what is now claimed by conveying with reasonable clarity to those skilled in the art that, as of the filing date, applicants were in possession of the invention. In view of the above explanations and evidence, Applicants respectfully request that the rejection of claims 11-13, 16 and 25-29 under 35 U.S.C. § 112, first paragraph, be reconsidered and withdrawn.

B. Availability of the Deposit

The Examiner has also rejected claims 11-13, 16 and 25-29 under 35 U.S.C. § 112, first paragraph, as allegedly failing to comply with the written description requirement. In particular, the Examiner has requested Applicants provide a statement assuring availability of the HACCI17 cDNA made under the Budapest Treaty. *See*, Paper No. 20061028, pages 11-12. To accommodate the Examiner's concern with respect to this matter, Applicants herein provide the following statement of assurance regarding public availability of the deposited HACCI17 cDNA:

Availability of the Deposited HACCI17 cDNA

Human Genome Sciences, Inc., the assignee of the present application, has deposited biological material under the terms of the

> Budapest Treaty on the International Recognition of the Deposit of Micro-organisms for the Purposes of Patent Procedure with the following International Depository Authority: American Type Culture Collection (ATCC), 10801 University Boulevard, Manassas, Virginia 20110-2209 (present address). The deposit was made on July 27, 1998, accepted by the ATCC, and given ATCC Accession Number 203071. In accordance with M.P.E.P. § 2410.01 and 37 C.F.R. § 1.808, assurance is hereby given that all restrictions on the availability to the public of ATCC Accession Number 203071 will be irrevocably removed upon the grant of a patent based on the instant application, except as permitted under 37 C.F.R. § 1.808(b). A partially redacted copy of the ATCC Deposit Receipt for Accession Number 203071 is enclosed herewith as Exhibit F.

In view of the above-provided assurance, Applicants respectfully request that the Examiner withdraw the rejection of claims 11-13, 16 and 25-29 under 35 U.S.C. § 112, first paragraph.

VI. Rejections Under 35 U.S.C. § 112, Second Paragraph

The Examiner has rejected claims 13 and 29 under 35 U.S.C. § 112, second paragraph for allegedly failing to set forth the subject matter, which applicants regard as their invention. Specifically, the Examiner alleges that claims 13 and 29 lack clear antecedent basis for the recitation of "isolated polypeptide". Applicants submit that claim 13 has been canceled herein and claim 25, from which claim 29 depends, has been amended herein, hereby rendering this rejection moot.

The Examiner has further rejected claim 13 as being indefinite. As mentioned above, Applicants have canceled claim 13 herein, rendering this rejection moot. Accordingly, Applicants respectfully request these rejections be reconsidered and withdrawn.

CONCLUSION

Applicants respectfully request that the above-made amendment and remarks be entered in the present application. In view of these remarks, Applicants request that the Examiner reconsider and withdraw each of the currently pending claim objections and rejections. The Examiner is invited to call the undersigned at the phone number provided below if any further action by Applicant would expedite the examination of this application.

If there any fees, not already accounted for, due in connection with the filing of this paper, please charge the fees to our Deposit Account No. 08-3425. If a fee, not already accounted for, is required for an extension of time under 37 C.F.R. § 1.136, such an extension is requested and the fee should also be charged to our Deposit Account.

Dated: FEB 1, 2007

Respectfully submitted,

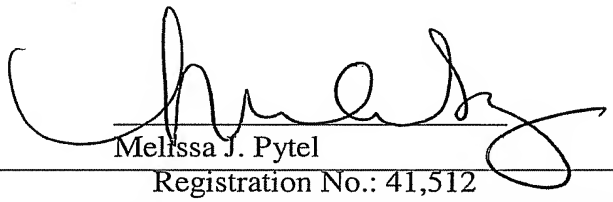

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EXHIBIT A

Expression of the cell adhesion molecules ICAM-1 and VCAM-1 in the cytosol of breast cancer tissue, benign breast tissue and corresponding sera

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Summary

Objective: Cellular adhesion molecules ICAM-1 and VCAM-1 have been implicated in tumor progression and metastasis. As the sequential interaction of neoplastic cells with the endothelium of tumor neovascularisation is believed to be essential for tumor metastasizing processes, we analysed the concentration of ICAM-1 and VCAM-1 in the cytosol of patients with human breast cancers and their corresponding sera. We compared the obtained values with established prognostic parameters for breast cancer. Benign breast tissues were also analyzed.

Patients and methods: Levels of ICAM-1 and VCAM-1 of 62 patients with invasive breast cancer and 17 patients with benign breast tissue were measured using commercially available sandwich enzyme-linked immunoassays with monoclonal antibodies. To establish a reference and control group, levels of ICAM-1 and VCAM-1 were measured in the sera of 66 women without breast tumors.

Results: The mean cytosol concentration of ICAM-1 and VCAM-1 was significantly higher in the breast cancer specimens than in the tissue of patients with benign breast diseases. This could be found not only in the tumor cytosol but also in the corresponding sera of the patients. No correlations between the ICAM-1 and VCAM-1 expressions and established prognostic parameters could be observed.

Conclusions: Our findings suggest that malignant breast cancer cells could induce neovascularisation with subsequent high expressions of ICAM-1 and VCAM-1. These upregulations of adhesion molecules might contribute to changes in invasive phenotypes by promoting endothelial cell adhesion and angiogenesis, as well as being responsible for the recognition of tumor cells by the human immune system. Prognostic relevance for the development of breast cancer could not be established.

Key words: Breast cancer; ICAM-1 and VCAM-1 molecules; Prognostic parameters.

Introduction

Intercellular adhesion molecule 1 (ICAM-1) and vascular cellular adhesion molecule 1 (VCAM-1) are both cytokine-inducible single-chain glycoproteins belonging to the immunoglobulin supergen family, with molecular weights of 90-114 kd and 105-110 kd, respectively [1-4]. These cellular adhesion molecules, which mediate homotypic and heterotypic cellular interactions, have been implicated in the various stages of tumor progression and metastasis [5]. A major role in the cell-cell interaction in inflammatory and immune responses is postulated for ICAM-1, which normally is found in endothelial cells, leukocytes and some epithelial tissues [6]. Rothlein *et al.* [7] and Seth *et al.* [8] described the existence of a soluble form of ICAM-1 in the circulation, with elevated levels being reported for several benign and malignant diseases. High levels have also been associated with liver metastasis in gastric, colon, gall bladder and pancreatic cancers [9].

VCAM-1 is also induced by endothelial cells mediating adhesion of lymphocytes and monocytes [10]. Banks *et al.* [10] reported that the soluble concentration of ICAM-1 and VCAM-1 levels in the sera of patients with different malignancies were significantly higher than in patients without malignancies. In patients with malignant melanoma it has been suggested that the expression of ICAM-1 is associated with tumor growth or even with a poorer overall survival rate [11, 12]. Fox *et al.* [13] postulated in 1995 that the tumor endothelium of breast cancer tissues displayed a significant heterogenity in the expression of adhesion molecules and that it was able to assume a pro-inflammatory phenotype. An upregulation of adhesion molecules might contribute to important changes in the invasive character of breast cancer cells by promoting endothelial cell adhesion and angiogenesis, as well as forming a substratum for tumor cells to assemble and attract macrophages. The role of ICAM-1 and VCAM-1 for the angiogenetic effects in patients with breast cancer has been described by Fox *et al.* [14].

Beyond the data published by Fox *et al.* [13, 14] only few data are available about the expression and significance of ICAM-1 and VCAM-1 in human breast cancer tissues. Liang *et al.* [15] found that patients with breast cancer and metastasis had higher ICAM-1 levels than those without metastasis and that shedding of ICAM-1 antigens from the tumor could block the attachment of

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cytotoxic T cells. He also postulated that a decreased number of cytotoxic T cells could lead to a loss in the inhibition of spreading tumor cells.

As breast cancer is one of the most typically lymphatic metastasizing tumors, the expression of ICAM-1 and VCAM-1 in the cytosol and the corresponding sera of women with invasive breast cancer was analyzed, to obtain data about the biological characteristics of this malignancy in comparison to benign breast tissues. Up to now, no data about the expression of ICAM-1 and VCAM-1 in the tumor cytosol of human breast cancers have been available. The results were compared with various established prognostic parameters for breast cancer such as tumor size, lymph node status, M-status, hormonal receptor status, histological grading, menopausal status and cathepsin-D status to examine if the expression of ICAM-1 and VCAM-1 in patients with breast cancer could be used as a prognostic parameter as postulated for human malignant melanoma.

The aim of our study was therefore to describe the expression of ICAM-1 and VCAM-1 in breast cancer tissue and the corresponding sera.

Patients and Methods

The concentration of ICAM-1 and VCAM-1 in the cytosol and in the sera of 62 patients with breast cancer and of 17 patients with benign breast tissues was measured with commercially available ELISA assays of BIERMANN (Biermann GmbH, Bad Nauheim, Germany). The patients were treated between 1994 and 1997 in the Department of Gynaecology of the University of Essen in Germany.

Twenty-for patients had T1 stage breast cancer, 20 T2 stage, 10 T3 stage and 8 T4 stage breast cancer. Thirty patients were nodal negative, 18 had N1 stage disease. 10 N2 and 4 N3 stage disease. Fifty-six patients had a primary M0 stage, whereas 6 patients had a primary M1 stage. Thirty-seven patients were estrogen and/or progesterone receptor positive.

To determine the normal concentration of ICAM-1 and VCAM-1, the sera of 66 women without any known disease were also examined. The applied assay is a so-called "Sandwich-ELISA", where monoclonal mouse antibodies are used.

After obtaining the tumor samples and the sera, ICAM-1 and VCAM-1 were determined as follows:

1) Washing of the plastic wells three times before starting the test with a washing solution containing a PBS buffer.

2) Incubation of the tumor cytosol and the sera of the patients and the control samples within the plastic wells, which contain

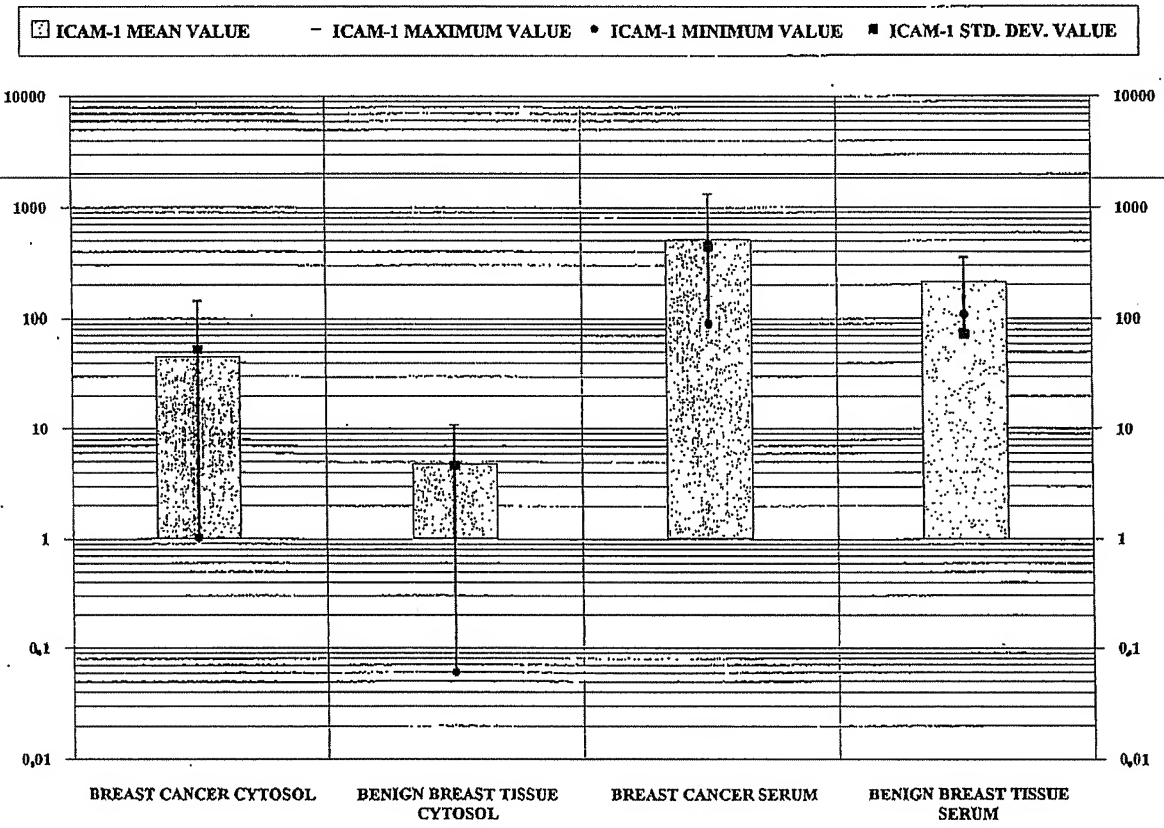

Figure 1 : ICAM-1 cytosol and ICAM-1 serum levels, expressed in ng/mg cytosol protein and ng/ml serum, respectively, of 62 patients with breast cancer and 17 patients with benign breast tissues. The differences between the patients with malignancies and those with benign breast tissues were, in both cases, statistically significant (p value < 0.05). Also statistically significantly higher ICAM-1 levels were observed in the sera of the patients in comparison to the corresponding tumor cytosols.

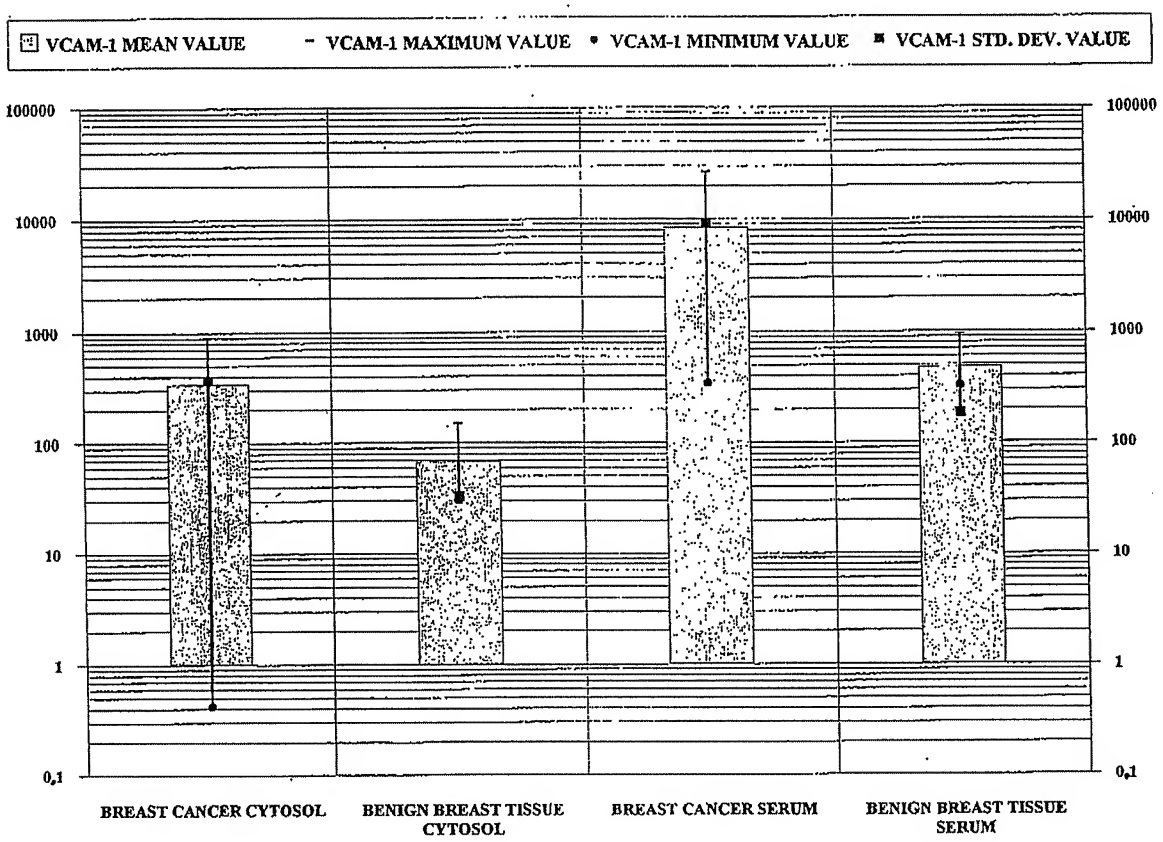

Figure 2 : VCAM-1 cytosol and ICAM-1 serum levels, expressed in ng/mg cytosol protein and ng/ml serum, respectively, of 62 patients with breast cancer and 17 patients with benign breast tissues. The differences between the patients with malignancies and those with benign breast tissues were, in both cases, statistically significant (p value < 0.05). Also statistically significantly higher VCAM-1 levels were observed in the sera of the patients in comparison to the corresponding tumor cytosols.

a monoclonal murine antibody that binds with the ICAM-1 and VCAM-1 molecules, for 60 minutes at 37°C in a humidity chamber.

3) Washing the plastic wells as described in step 1.

4) Incubation of the plastic wells for 30 minutes at 37 °C in a humidity chamber with the second antibody. This antibody binds with the complex of the primary antibody and the ICAM-1 and VCAM-1 molecules. It is a peroxidase conjugated goat anti hyman IgG antibody that acts as the detector.

5) Washing the plastic wells as described in step 1.

6) Incubation of the probes with a substrate solution containing a substrate buffer and a TMB solution for 30 minutes at room temperature in a dark chamber.

7) Stopping of the enzymatic reaction by incubating the wells with a 2 N HCL solution.

8) Measuring the photometric extinction of the probes at 450 nm for 30 minutes in a spectrophotometer.

By this method quantitative results were obtained and presented in ng/mg cytosol protein for the tumor samples and as ng/ml for the sera.

By analyzing standards of known ICAM-1 and VCAM-1 concentrations coincident with the samples and plotting a curve of signal versus concentration, the concentration of unknowns was determined. The standards consisted of 6 vials of lyophilised recombinant soluble ICAM-1 and VCAM-1.

Estrogen and progesterone receptors were analyzed with the DCC-(dextran-coated-charcoal) technique (5 point radioligand assay) described by Scatchard [16]. Protein concentration was determined by the method of Lowry [17]. Five hundred mg of fresh frozen tissue were needed to evaluate the hormonal receptor status.

The cut-off point between receptor positive and receptor negative was 10 fmol/mg cytosol protein; i. e. hormonal receptor concentrations of higher than 9 fmol/mg cytosol protein were considered as positive.

The cathepsin-D status was evaluated in the tumor cytosol using an immunoradiometric assay (ELSA-CATH-D of CIS bio international; GIF-sur-YVETTE CEDEX, France). The cut-off point between cathepsin-D positive and negative was 30 fmol/mg cytosol protein.

Statistical analyses

Statistical analyses were done with the SAS (SAS Institute, NC [18]) program. The student's t test and the Wilcoxon rank sum test for paired and/or independent distributed samples were also used. Significant correlations were given at values of $p < 0.05$. The Pearson correlation coefficient for the examined parameters was also used.

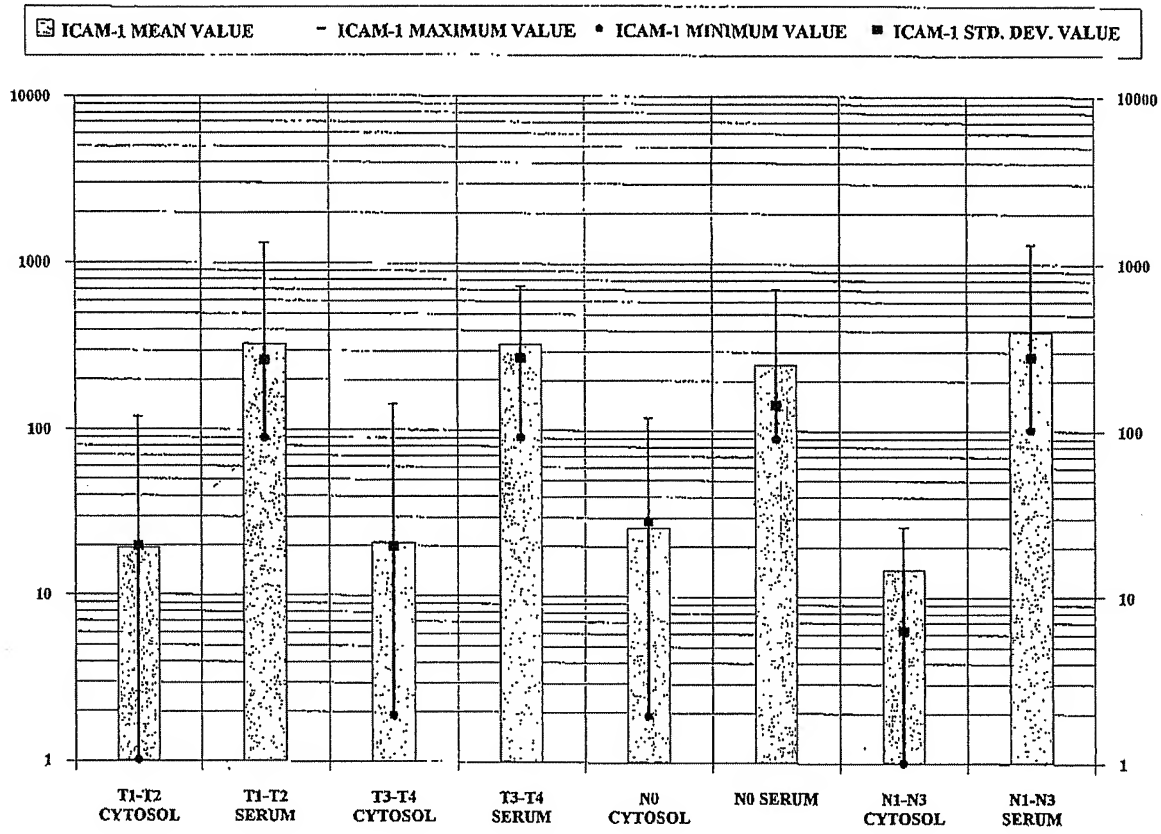

Figure 3 : Correlation between the mean ICAM-1 cytosol and serum levels, expressed in ng/mg cytosol protein and ng/ml serum, respectively, and the tumor and nodal status of the 62 patients with breast cancer. No statistically significant differences between T1-T2 and T3-T-4 or between N0 and N1-N3 tumors and the corresponding ICAM-1 levels whether in the tumor cytosol or in the sera could be observed (p values always > 0.05).

Results

General results

Concentration of soluble ICAM-1 and VCAM-1 was found in the sera of our control group, the patients with benign breast tissues and the patients with breast cancer. ICAM-1 and VCAM-1 was also present in the cytosol of the 17 benign cases and the 62 patients with a primary breast cancer.

In our reference group of 66 patients without breast tumors (aged between 23 and 51 years) the mean ICAM-1 serum concentration was 203.05 ng/ml, whereas the mean VCAM-1 value was 1466ng/ml. The maximun value for ICAM-1 was 533.4 ng/ml, the minimum value 85.7 ng/ml; the maximum value for VCAM-1 was 13196 ng/ml and the minimum value was 213.3 ng/ml.

When comparing the group of patients with benign breast tumors with those of breast cancers we could find statistically significant differences. The sera and the tumor cytosols of the breast cancer patients contained higher ICAM-1 and VCAM-1 values than the cytosols and sera of the corresponding patients with benign disease. The mean ICAM-1 serum value for the patients with a breast cancer was 506.08 ng/ml (in benign tissues a mean value of 216.89 ng/ml was observed). In the tumor cytosol the values were 45.65 ng/mg protein and 4.84 ng/mg protein, respectively. The mean VCAM-1 serum level was 8432.7 ng/ml in the patients with breast cancer and 479.33 ng/ml in the patients with benign tissues, whereas in the tumor cytosols the concentration was 340.9 ng/mg protein and 67.49 ng/mg protein, respectively. All the paired and independent Student's t tests were statistically significant (p values < 0.05). Figures 1 and 2 show the differences of the mean, maximum, minimum and standard deviation values of ICAM-1 and VCAM-1 between the patients with benign and malignant tissues.

No correlations between the ICAM-1 and VCAM-1 expressions in the sera and the tumor cytosols to the tumor size, nodal status, metastasis status, tumor grading, menopausal status, hormonal receptor status and to the cathepsin-D status could be observed (p values always > 0.05).

ICAM-1 expression in patients with breast cancer and benign breast tissues

Patients with a primary breast cancer (62 cases) showed higher levels of ICAM-1 in the sera and in the

tumor cytosols in comparison to the 17 patients who underwent surgery because of benign breast diseases. The mean serum level of ICAM-1 was evaluated on the same day of surgery, just before surgery. and in the cytosol of the corresponding tissues.

The mean serum level for ICAM-1 in patients with breast cancer was 506.08 ng/ml (std.dev. 443.18 ng/ml) whereas in the sera of the patients with benign tumors the mean values for ICAM-1 betrayed 216.89 ng/ml (std. dev. 72.98 ng/ml). The maximum value for ICAM-1 in benign tissues was 353.84 ng/ml and the minimum value was 108.83 ng/ml; in malignant tissues the values were 1311 ng/ml and 89.45 ng/ml, respectively. In the cytosol the mean value of breast cancer tissues was 45.65 ng/mg protein (with a std. dev. of 52.71 ng/mg protein) in contrast to 4.85 ng/mg protein in benign tissues (std. dev. 4.69 ng/mg protein). The maximum value for ICAM-1 in breast cancer was 142.74 ng/mg protein, the minimum value 1.01 ng/mg protein (in benign tissue values of 10.87 ng/mg protein and 0.06 ng/mg protein were found. All the differences were statistically significant.

The serum levels of the patients with benign breast tumors were similar to those of our control group (216.89 ng/ml and 203.05 ng/ml respectively).

VCAM-1 expression in patients with breast cancer and benign breast tissues

The mean serum level for VCAM-1 in patients with breast cancer was 8432.70 ng/ml (std. dev. 9424.80 ng/ml) versus 479.33 ng/ml (std. dev. 182.91) in the group of patients with benign breast tumors. The maximum value for the breast cancer group was 26827 ng/ml; the minimum value was 342.56 ng/ml. In the benign tissue group the maximum value was 945.19 ng/ml and the minimum value was 323.88 ng/ml. In the tumor cytosol the values for VCAM-1 were 340.91 ng/mg protein (std. dev. 372.77 ng/mg protein) in the patients with breast cancer and 67.49 ng/mg protein (std. dev. 32.97 ng/mg protein) in the patients with benign tumors. The maximum values were 900.4 ng/mg protein and 149.63 ng/mg protein respectively; the minimum values were 0.43 ng/ml protein and 30.76 ng/mg protein, respectively. All the differences were statistically significant.

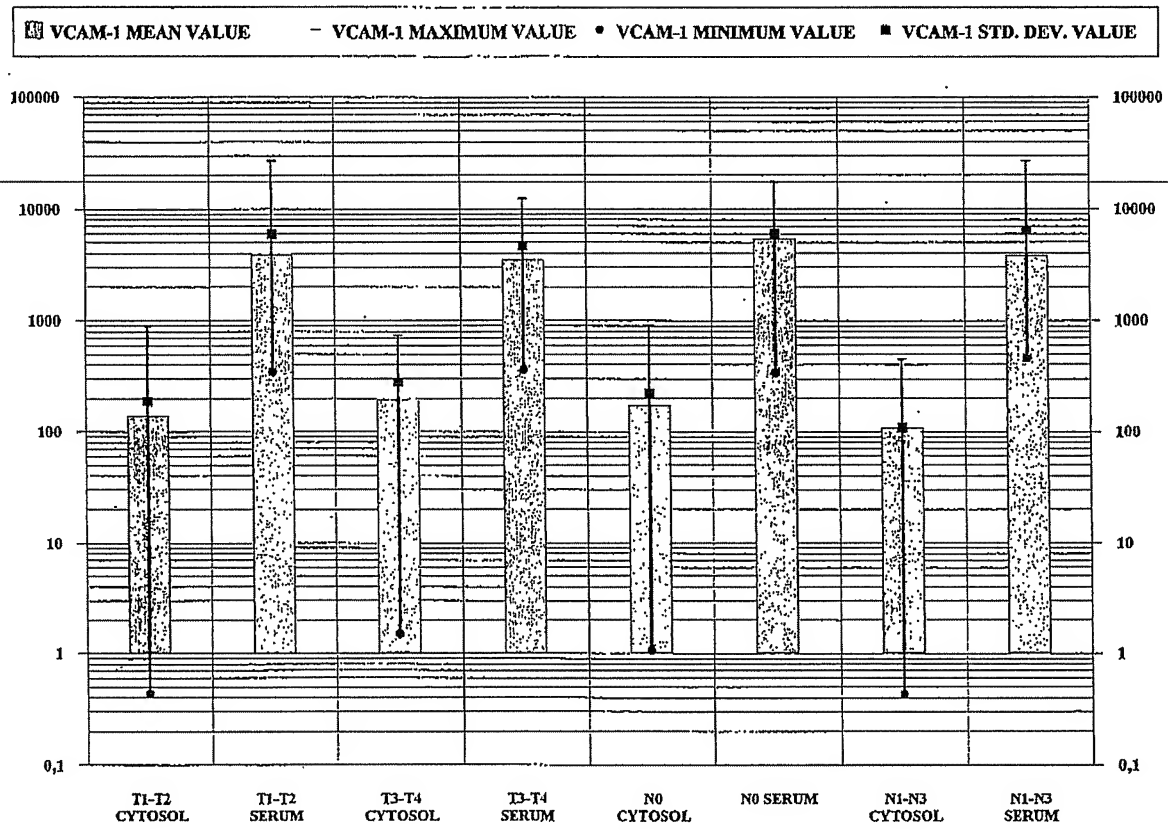

Figure 4 : Correlation between the mean VCAM-1 cytosol and serum levels, expressed in ng/mg cytosol protein and ng/ml serum, respectively, and the tumor and nodal status of the 62 patients with breast cancer. No statistically significant differences between T1-T2 and T3-T-4 or between N0 and N1-N3 tumors and the corresponding VCAM-1 levels whether in the tumor cytosol or in the sera could be observed (p values always > 0.05).

When comparing the serum levels of our control group with the levels of the patients with benign breast tumors no statistically significant differences could be observed. The mean value of the control group was 456.22 ng/ml (std. dev. 154.73 ng/ml) whereas, as stated above, the mean serum value of our benign breast tissue group was 479.33 ng/ml (std. dev. 182.91).

ICAM-1 and VCAM-1 expression in patients with breast cancer and the correlation to established prognostic parameters

When comparing the ICAM-1 and VCAM-1 levels of the patients with T1-T2 stage breast cancer with those with T3-T4 stage breast cancer no statistically significant correlations could be observed (mean serum level for ICAM-1 for T1-T2 stage patients = 329.72 ng/ml (std. dev. 262.33 ng/ml) and mean cytosol level = 19.33 ng/mg cytosol protein with a std. dev. of 20.01 ng/mg cytosol protein versus 331.61 ng/ml sera (std. dev. 273.15 ng/ml) and 20.93 ng/mg cytosol protein (std. dev. 19.91 ng/mg cytosol protein) in patients with T3-T4 breast cancers. For the adhesion molecule VCAM-1 similar results were obtained. No correlation between the obtained values of the tumor cytosol or the corresponding sera and the tumor stage could be observed.

In the same way no differences between the nodal negative or positive patients could be described. The mean serum level of the nodal negative patients for ICAM-1 was 251.31 ng/ml (std. dev. 142.73 ng/ml) and for VCAM-1 5381.61 ng/ml (std. dev. 5987.44 ng/ml). In the cytosol the values were as follows: ICAM-1 : 26.16 ng/mg cytosol protein (std. dev. 28.56 ng/mg cytosol protein) and for VCAM-1 173.86 ng/mg cytosol protein (std. dev. 221.31 ng/mg cytosol protein). The nodal positive patients showed similar expression of ICAM-1 and VCAM-1. No statistically significant differences could be observed when comparing the hormonal receptor status, the cathepsin-D status, tumor grading or menopausal status with the obtained ICAM-1 and VCAM-1 levels whether in the sera of the patients or in the tumor cytosols (figures 3 and 4 depict the distribution of ICAM-1 and VCAM-1 and the tumor and nodal status of the 62 breast cancer patients).

Discussion

This is one of the first reports to describe the distribution patterns of ICAM-1 and VCAM-1 in the cytosol of benign and malignant breast tissues and in their corresponding sera. The ICAM-1 and VCAM-1 concentrations of the breast cancers were elevated compared with benign tissues.

There data hereby confirm earlier reports by Tsujisaki et al. [9] and Harning *et al.* [12] where such elevated ICAM-1 and VCAM-1 levels in breast cancer tissues were described.

The interpretation of these results in relation to the clinical and biological significance remains complicated by the fact that though having analysed homogeneous groups, patients with breast cancer had varying stages of disease and some had concomitant diseases with inflammatory reactions.

The differences in the findings with regard to ICAM-1 and VCAM-1 probably reflect differences in source, kinetics of expression or destruction, and possible signals inducing their expression and/or release. As ICAM-1 and VCAM-1 are expressed in the endothelium of tumor associated vessels, our results confirm the data of Fox *et al.* who found, using immunohistochemistry, higher levels of CAM's in the tumor-associated vessels in comparison to benign tissues. Therefore, angiogenesis can be seen as the motor for the high expression of CAM's. It is known that VCAM-1 is induced in the endothelium of the tumor-associated vessels by TNF-alpha, IL-1ß, y-interferon and IL-4 [19-21]. ICAM-1 expression has been reported to be increased by IL-1, y- and ß-interferon, TNF-alpha, IL-4, IL-6, and IL-2 with effects being tissue dependent [11, 22, 23]. Whether the cytokines responsible for the induction of CAM expression and shedding are tumor-derived or derived from surrounding vascular endothelia remains unclear. The mechanism underlying shedding of adhesion molecules is not yet understood completely. Banks *et al.* [10] postulated that the shedding of adhesion molecules could have profound implications for tumor metastasis as the shedding of ICAM-1 and VCAM-1 by circulating tumor cells could allow them to escape to the surveillance of cytotoxic T-cells and natural killer cells and therefore allow metastasis. In the same way it can be speculated that the increased ICAM-1 and VCAM-1 levels in malignant tumor cytosol, reflected also in the corresponding sera, could escribe the ability of breast cancer cells to block the attachment of cytotoxic T-cells by creating a neovascularisation of the surrounding blood vessels. The subsequent decreased number of cytotoxic T-cells could be a reason for the failure of the immune response in inhibiting the spread of cancer cells. Liang *et al.* [15] found that patients with breast cancer and metastases had a lower percentage of CD8 lymphocyte subsets and that these patients had higher levels of circulating ICAM-1 molecules. These observations remain, nevertheless, speculative as the immune responses of patients with breast cancer may be different between the metastatic and non-metastatic status.

As we could not find statistically significant differences between T1-T2 tumors and T3-T4 tumors or between the nodal status or the M status of our breast cancer patients, we conclude that CAM expression is not tumor dependent but only dependent on the tumor-associated endothelial expression of the surrounding vessels. This could also explain the findings of Fox *et al.* where the expression of PECAM, ICAM-1, E- and P-selectin was observed dominantly at the tumor periphery [14].

ICAM-1 and VCAM-1 can therefore be considered as a product of tumor-induced angiogenesis and not as tumor cell born molecules. Thus, they are not suitable for use as prognostic parameters. In patients without other angiogenetic developing diseases ICAM-1 and VCAM-1 can nevertheless be used as possible markers for determining the angiogenetic potency of breast cancer tumors.

Further long-term follow-up studies and observations

about the recurrence-free and overall survival rates of patients with breast cancer have to be performed to evaluate whether angiogenetic rates of the vascular endothelium surrounding the cancer tissues are correlated with better or poorer disease-free or overall survival rates, and whether patients with higher tumor-associated angiogenetic rates need or do not need adjuvant polychemotherapy.

Acknowledgement

We wish to acknowledge the technical assistance of Siegrid Arndt.

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EXHIBIT B

Angiogenesis mediated by soluble forms of E-selectin and vascular cell adhesion molecule-1

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ENDOTHELIAL adhesion molecules facilitate the entry of leukocytes into inflamed tissues. This in turn promotes neovascularization, a process central to the progression of rheumatoid arthritis, tumour growth and wound repair[1]. Here we test the hypothesis that soluble endothelial adhesion molecules promote angiogenesis[2–4]. Human recombinant soluble E-selectin and soluble vascular cell adhesion molecule-1 induced chemotaxis of human endothelial cells *in vitro* and were angiogenic in rat cornea. Soluble E-selectin acted on endothelial cells in part through a sialyl Lewis-X-dependent mechanism, while soluble vascular cell adhesion molecule-1 acted on endothelial cells in part through a very late antigen (VLA)-4 dependent mechanism. The chemotactic activity of rheumatoid synovial fluid for endothelial cells, and also its angiogenic activity, were blocked by antibodies to either soluble E-selectin or soluble vascular cell adhesion molecule-1. These results suggest a novel function for soluble endothelial adhesion molecules as mediators of angiogenesis.

First, we showed that recombinant human soluble E-selectin stimulated chemotaxis of human umbilical vein endothelial cells (HUVECs) (Fig. 1*a*) and human dermal microvascular endothelial cells (HMVECs) (Fig. 1*b*). Soluble E-selectin was potently chemotactic for HUVECs in the picomolar range, with 0.01 pM soluble E-selectin inducing chemotaxis equivalent to 60 nM control angiogenic cytokine basic fibroblast growth factor (bFGF). Recombinant human soluble vascular-cell-adhesion molecule-1 (soluble VCAM-1) was also chemotactic, with 1 nM soluble VCAM-1 inducing HUVEC chemotaxis equivalent to 60 nM bFGF (Fig. 1*c*). Similar results were obtained with HMVECs (Fig. 1*d*), indicating that these effects were not restricted to HUVECs. Checkerboard analysis, incorporating varying concentrations of chemoattractant in the upper and lower chemotaxis chambers, indicated that the effects of soluble

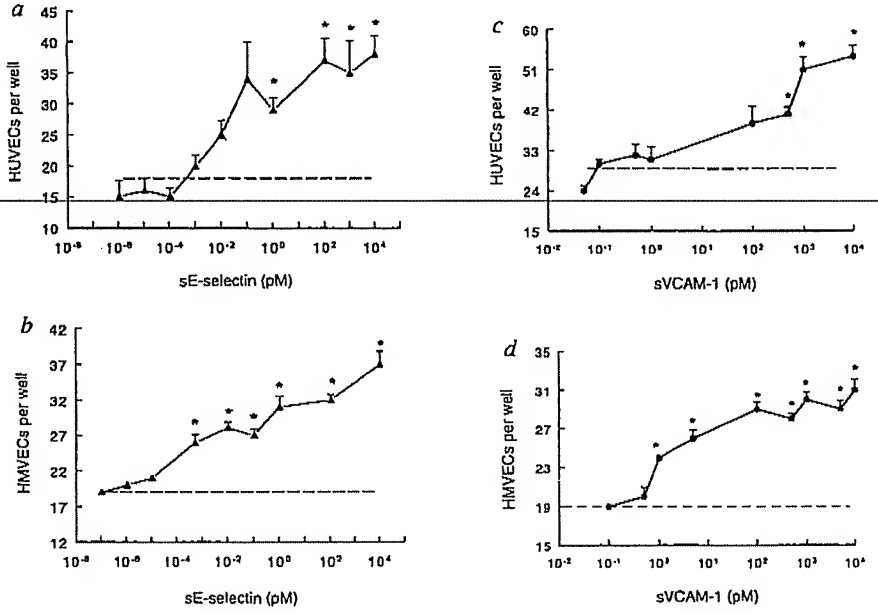

FIG. 1 Chemotaxis of endothelial cells. Chemotaxis of HUVECs or HMVECs (Clonetics, San Diego) was performed in 48-well blind-well chemotaxis chambers with polycarbonate membranes of 8 μm pore size (Neuroprobe, Cabinjohn, MD)[5,17]. HUVECs (2.5×10^4 cells per well) or HMVECs (3.75×10^4 cells per well) in 25 μl of Roswell Park Memorial Institute (RPMI) media containing +0.1% fetal calf serum were added to the bottom wells of the chambers. Inverted chambers were incubated at 37 °C for 2 h, allowing endothelial cell attachment. The following were added to the top half of reinverted chambers: PBS with or without recombinant human soluble E-selectin (sE-selectin) (*a*, *b*), or soluble VCAM-1 (s-VCAM-1) (*c*, *d*) (Biogen, Cambridge, MA), or bFGF (60 nM) (R&D Systems, Minneapolis). The sE-selectin was biologically active, functioning as an adhesion molecule[2]. This binding was blocked by EDTA (R. Lobb, personal communication). After incubation for 2 h, the membranes were removed, fixed in methanol and stained with Diff-Quik (Baxter Diagnostics, Chicago). Each test group was assayed in quadruplicate. Three high-power microscope fields were counted in each replicate well and results were expressed as cells per well. Statistical analysis was done with an unpaired Student's *t*-test without correcting for multiple comparisons. Values significantly different ($P<0.05$) from the PBS control (horizontal broken line) are indicated by stars. Chemotaxis in response to bFGF was: *a*, 25±2 cells per well (±s.e.m.); *b*, 37±2; *c*, 48±1; *d*, 31±1. Endotoxin concentrations in the soluble adhesion preparations did not exceed 0.04 EU mg^{-1} in the limulus amebocyte lysate assay (Associates of Cape Cod, Woods Hole, MA). PBS controls containing up to 0.4 EU mg^{-1} endotoxin did not affect the assay. Results represent 1 of at least 3 experiments. *e*, *f*, The results of representative checkerboard analyses using HUVECs and varying concentrations of sVCAM-1 or sE-selectin in the upper or lower compartment of the chemotaxis chamber[20]. Both sE-selectin (*e*) and sVCAM-1 (*f*) induced chemotactic rather than chemokinetic responses. Results are expressed as the number of cells ±s.e.m. per replicate well.

e

	Upper			
Lower	0	0.5 pM	110 pM	1.1 nM
0	19.3 ± 0.2	23.0 ± 1.2	28.5 ± 1.1	27.3 ± 2.1
0.5 pM	19.3 ± 0.7	20.8 ± 1.2	22.5 ± 1.1	27.3 ± 1.4
110 pM	17.3 ± 0.2	22.0 ± 1.7	26.3 ± 0.8	25.0 ± 1.6
1.1 nM	16.5 ± 1.2	22.3 ± 1.5	24.3 ± 1.0	22.8 ± 0.4

f

	Upper			
Lower	0	100 pM	1 nM	5 nM
0	13.3 ± 0.7	16.0 ± 1.1	25.8 ± 1.6	29.0 ± 2.8
100 pM	13.0 ± 0.7	11.8 ± 0.5	21.0 ± 1.1	28.5 ± 1.7
1 nM	12.8 ± 1.2	14.0 ± 0.9	13.3 ± 1.1	27.5 ± 2.2
5 nM	11.7 ± 0.5	14.3 ± 0.7	12.5 ± 0.4	12.3 ± 0.2

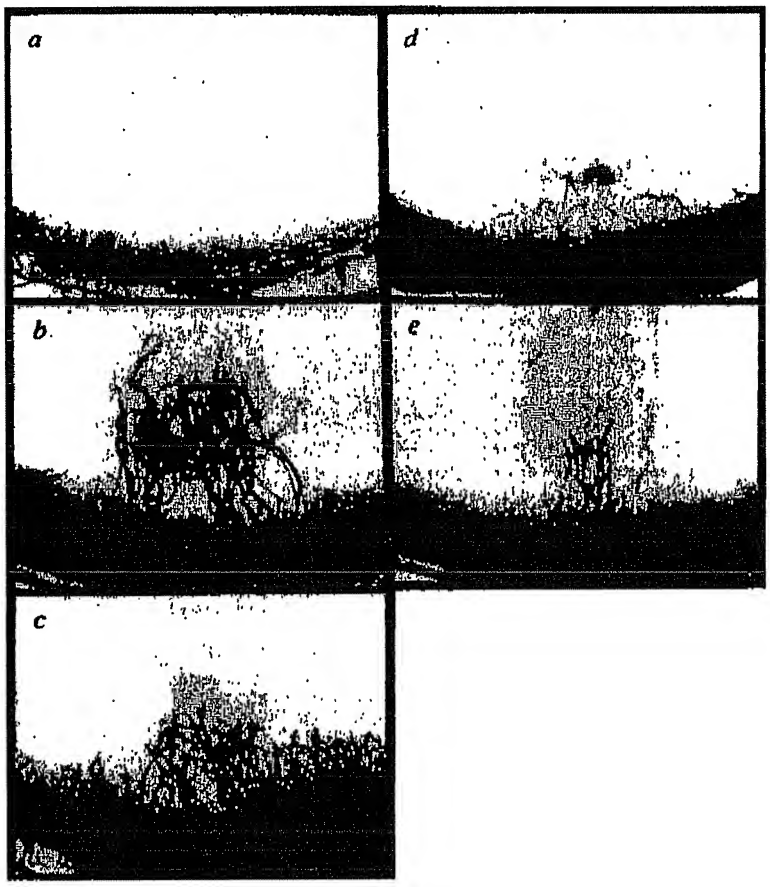

FIG. 2 *a*, Negative angiogenic response induced control PBS; *b*, positive angiogenic response induced by soluble E-selectin (10 nM); *c*, positive angiogenic response induced by soluble VCAM-1 (10 nM); *d*, minimal angiogenic response induced by rheumatoid arthritis (RA) synovial fluids (SFs) incubated with anti-E-selectin; *e*, markedly suppressed angiogenic response induced by RA SFs incubated with anti-VCAM-1.
METHODS. Test substances were combined 1:1 with Hydron (Interferon Sciences, New Brunswick, NJ) and implanted into the normally avascular corneal stroma of the rat several mm from the limbus[5,21]. Corneas were perfused with colloidal carbon after 7 days to provide a permanent record of the angiogenic response (33×). No cornea exhibited histological evidence of nonspecific inflammation. All human samples were obtained with Institutional Review Board approval. SFs were obtained from arthrocentesis of patients with RA. SFs were diluted 1:50 with PBS and incubated with either anti-E-selectin, anti-VCAM-1, or isotype-matched control monoclonal antibody (mAb) for 1 h at 37 °C. The mAbs used were: mAb BB11, which recognizes E-selectin; mAb 4B9, which recognizes VCAM-1 domain 1; and mAb GH12, which recognizes VCAM-1 domain 4 (refs 22, 23). All test mAbs were used at 10 μm ml^{-1} and obtained from Biogen (Cambridge, MA). Control mAbs (10 μl ml^{-1}) were obtained from Coulter Diagnostics (Hialeah, FL); mAbs 4B9 and GH12 were used simultaneously.

E-selectin or soluble VCAM-1 were chemotactic, not chemokinetic, for HUVECs or HMVECs (Fig. 1*e*, *f*). Neither soluble adhesion molecule (SAM) induced mitogenesis of HUVECs or HMVECs, suggesting that they mediated angiogenesis independently of stimulating endothelial cell proliferation (results not shown).

We next determined whether soluble E-selectin and soluble VCAM-1 were angiogenic *in vivo*. SAMs were incorporated into Hydron pellets and implated into rat corneas. Soluble E-selectin (10 nM) or soluble VCAM-1 (10 nM) induced an angiogenic response in 3 out of 4 and 4 out of 4 corneas, respectively (Fig. 2*a–c*).

To determine whether the SAMs were acting *in vivo* by inducing the release of angiogenic cytokines by HUVECs[5], these cells were incubated with either soluble E-selectin (up to 1.1 nM) or soluble VCAM-1 (up to 50 nM), at concentrations that met or exceeded those necessary to induce HUVEC chemotaxis *in vitro*. Treatment of HUVECs with these SAMs did not increase their production of interleukin-8 (IL-8), tumour necrosis factor-α (TNF-α), or bFGF, as measured by enzyme-linked immunosorbent assay (ELISA), indicating that they did not act as indirect angiogenic mediators (results not shown).

To investigate the mechanism of action of soluble E-selectin on endothelial cells, HUVECs were incubated with a monoclonal antibody to the E-selectin ligand, sialyl Lewis-X, which is expressed on a variety of cells, including endothelial cells[6,8] (Fig. 3). We detected the sialyl Lewis-X antigen on HUVECs by immunoperoxidase histochemistry (results not shown). In the presence of anti-sialyl Lewis-X, chemotaxis of HUVECs in response to soluble E-selectin was significantly reduced ($P<0.05$), indicating that soluble E-selectin induces HUVEC chemotaxis in part through binding endothelial sialyl Lewis-X. Similar experiments involved incubating HUVECs with monoclonal antibodies to the soluble VCAM-1 ligand VLA-4, which

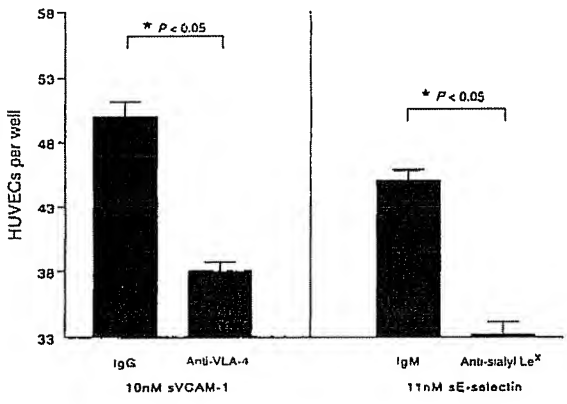

FIG. 3 Soluble E-selectin and soluble VCAM-1 mediate HUVEC chemotaxis by sialyl Lewis-X and VLA-4-dependent mechanisms, respectively.
METHODS. We determined whether HUVECs migrated in response to sE-selectin or sVCAM-1 when the ligands for these molecules on target HUVECs were blocked. RPMI (0.5 ml) containing 10% fetal calf serum and 10 μg ml^{-1} mAb or isotype-matched control mAb were placed in replicate wells of confluent HUVEC-containing 24-well plates. The mAbs used were: mAb CSLEX1, which recognizes sialyl Lewis-X (Le^x) (Becton Dickinson, San Jose, CA); and mAb HP 1/2, which recognizes the α chain of VLA-4 (Biogen)[24]. Isotype-matched control mAbs were obtained from Coulter Diagnostics. After incubation for 1 h at 37 °C in a humidified incubator gassed with 5% CO_2, HUVECs were collected and resuspended in RPMI containing 0.1% fetal calf serum and 10 μg ml^{-1} mAb, and chemotaxis was performed as described in Fig. 1. Results represent 1 of 3 experiments, each performed in quadruplicate. Additionally, in 3 experiments, concentrations of sE-selectin down to 1.1 nM or sVCAM-1 down to 5 pM showed similar results. Statistical analysis was done with an analysis of variance[25].

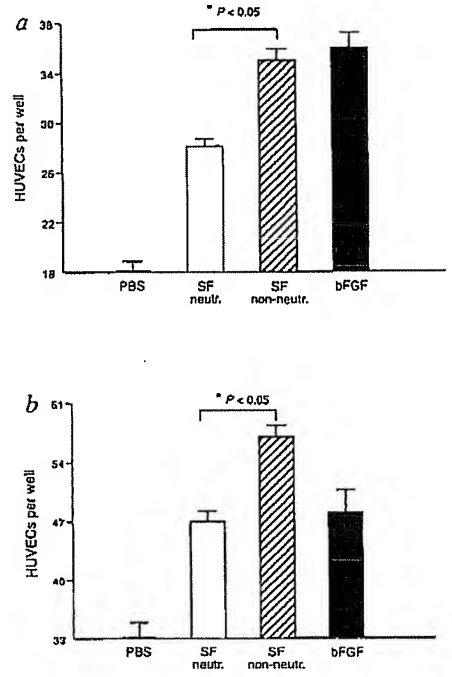

FIG. 4 Soluble E-selectin and soluble VCAM-1 account for a large portion of the RA SF chemotactic activity for HUVECs.
METHODS. RA SFs were obtained from 9 patients, diluted 1:50 with PBS and depleted of sE-selectin or sVCAM-1 as described in Fig. 2. Chemotaxis with SFs was done as described in Fig. 1. SF neutr., neutralized RA SFs; SF non-neutr., SFs incubated with isotype-matched control mAbs. Results represent the means±s.e.m. for 9 patients. Depleted SFs contained no detectable antigenic sE-selectin or sVCAM-1 as measured by ELISA (R&D Systems, Minneapolis) (results not shown). Anti-E-selectin (*a*) or anti-VCAM-1 (*b*) completely eliminated the chemotactic activity for HUVECs induced by 11 nM sE-selectin or 10 nM sVCAM-1, respectively. None of the mAbs had any effect on bFGF-induced HUVEC chemotaxis.

is also expressed on endothelial cells and was detected on HUVECs by immunoperoxidase histochemistry (results not shown)[9]. Incubation of HUVECs with anti-VLA-4 significantly diminished ($P<0.05$) the chemotactic activity for HUVECs in response to soluble VCAM-1, indicating that soluble VCAM-1 was acting on HUVECs to induce their chemotaxis in part by a VLA-4-dependent mechanism (Fig. 3).

We then determined whether SAMs contributed to the angiogenic activity of rheumatoid synovial fluid. Incubation with anti-E-selectin or anti-VCAM-1 significantly attenuated the rheumatoid synovial fluid chemotactic activity for HUVECs (Fig. 4*a*, *b*). Rheumatoid synovial fluid (5 μg protein) was potently angiogenic in all 3 corneas examined. Neutralization of soluble E-selectin or soluble VCAM-1 diminished or completely abolished the angiogenic response (1 out of 11 positive corneas (Fig. 2*d*), and 2 out of 14 positive corneas and 3 out of 14 more or less weakly positive corneas (Fig. 2*e*), respectively). Incubation with control isotype-matched monoclonal antibody had no effect on the angiogenic activity present in the synovial fluid (6 out of 6 positive corneas). These results indicate that soluble E-selectin and soluble VCAM-1 account for a large portion of the angiogenic activity found in rheumatoid synovial fluids.

Cellular adhesion and angiogenesis, often regarded as separate processes, may be linked. We have shown that the β3 integrin subunit is expressed in rheumatoid arthritis angiogenesis-rich synovial tissue, but virtually absent in normal angiogenesis-deficient synovial tissue[10]. Interestingly, the adhesion molecule αvβ3 integrin has recently been identified as a marker of angiogenic vascular tissue in wound granulation tissue[11]. Paradoxically, addition of anti-αvβ3 promoted tube formation in fibrin gels and inhibited endothelial cell proliferation on a fibrinogen matrix[12]. Anti-α2β1 integrin enhanced the number of capillary tubes formed by HUVECs *in vitro*[12]. Similarly, sialyl Lewis-X/A has been implicated in bovine capillary morphogenesis *in vitro*[13].

It has been shown that E-selectin and VCAM-1 are upregulated both in rheumatoid synovial fluid *in situ* and in soluble form in synovial fluid from rheumatoid arthritis compared with osteoarthritis[4,14–16]. Despite the increased quantities of SAMs in angiogenic disease states, their main known functions have included mediating cellular adhesion when immobilized to plastic, and, for soluble E-selectin, recruiting neutrophils *in vitro*[2,3]. We have shown that soluble E-selectin and soluble VCAM-1 are angiogenic in the 10 nM range in the cornea. This compares with amounts reported for the induction of angiogenesis by IL-8, TNF-α, aFGF and bFGF, angiotropin, angiogenin and vascular endothelial growth factor[5,17–19]. The studies described here suggest a proangiogenic role for soluble E-selectin and soluble VCAM-1. It is possible that when leukocytes bind to endothelial cells with concomitant release of cytokines, these molecules expressed on endothelial cells are then shed. The shed molecules in turn bind adjacent endothelial cells via their respective ligands, sialyl Lewis-X and VLA-4, exert a direct angiogenic effect on these endothelial cells and mediate inflammation. Our results therefore demonstrate a link between cellular adhesion and angiogenesis, and suggest a novel function for soluble E-selectin and soluble VCAM-1 in angiogenesis. □

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EXHIBIT C

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The prognostic value of quantitative angiogenesis in breast cancer and role of adhesion molecule expression in tumor endothelium

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Key words: adhesion molecules, angiogenesis, immunohistochemistry, prognosis, selectins, tumor vascularity

Summary

Angiogenesis is the formation of new capillaries from the existing vascular network and is essential for tumor growth and metastases. Increased microvessel density in breast cancer is associated with lymph node metastasis and reduced survival. We have assessed tumor vascularity in 211 breast carcinomas using a more rapid technique based on a Chalkley point eyepiece graticule. We confirmed using this method a significant reduction in overall survival between patients stratified by Chalkley count in both a univariate (p=0.02) and multivariate (p=0.05) analysis.

Since studies have suggested that cell adhesion molecules (CAMs) might be important in the angiogenic process, and interaction of neoplastic cells with this neovasculature is a significant step in tumor metastasis, we have also examined the expression of CAMs in a subset of these tumors (n=64). Using immunohistochemistry we observed widespread and intense staining on the endothelium of tumor-associated vessels for PECAM (100%), ICAM 1 (69%), and E- and P-selectins (52% and 59% of cases respectively). Endothelial expression of the selectins was more prominent at the tumor periphery. Immunoreactivity of ICAM-1 (34%), PECAM (1.6%), and E- and P-selectins (7% and 37% of cases respectively) was also observed on the neoplastic element of the tumors.

Introduction

Angiogenesis is the formation of new capillaries from the existing vascular network and is essential for tumor growth [1]. It is a complex multistep process involving extracellular matrix remodelling, endothelial cell migration and proliferation, capillary differentiation, and anastomosis [2]. Studies have shown that increased microvessel density (used as a measure of angiogenesis) is associated with lymph node metastasis in breast cancer [3-9]. The metastatic process is likely to be dependent on specific interactions with both extracellular matrix and endothelium [10,11]. This will be governed by the expression of cell adhesion molecules (CAM) and their co-ordinate ligands on tumor endothelium such as the integrins, immunoglobulin (Ig) superfamily members, and selectins. The expression of integrins on breast tumors has been examined [12,13], but

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Table 1. Clinicopathological characteristics of patients and tumors

Patient characteristic	Number
Age median (range)	57 (28-83) years
<50 years	61
≥50 years	150
Surgical treatment	
Simple mastectomy	56
Lumpectomy	155
Adjuvant treatment	
Chemotherapy	49
Tamoxifen	109
Lymph nodes	
0	112
1-3	72
≥4	27
Tumor size median (range)	2.25 (0.8-8) cm
<2 cm	66
≥2 cm	145
Histology	
Ductal	157
Lobular	27
Others	27
Grade	
I	17
II	80
III	60
ER* median (range)	19.8 (0-604)
<10	78
≥10	133
EGFR* median (range)	16.4 (0-210)
<20	119
≥20	92
Survival follow-up	
median (range)	42 (9-62) months
Deaths	27
Recurrences	44

* fmol/mg protein

only limited data on the Ig superfamily and selectins is available for cultured breast tumor cell lines [14]. Thus, since there are no reports of their expression on breast carcinomas in vivo, we have examined the pattern of CAM expression in breast tumors.

In addition to phenotyping breast tumor endothelium we have used quantification of the neovasculature to examine the effect of tumor vascularity on the prognosis of breast cancer patients. In a previous issue of this journal we demonstrated in 109 node negative patients that those with highly vascular tumors, as measured by the rapid method of Chalkley counting, have a significantly shorter relapse-free and overall survival [9]. In this issue we present findings in 211 patients with a longer follow up and also examine 64 of these tumors for the frequency and pattern of expression of the selectin and immunoglobulin families of cellular adhesion molecules.

Materials and methods

Tumors and patients

Consecutive series of primary breast tumor samples (n=211) were collected from the archival files of the Pathology Department of the John Radcliffe Hospital. Sixty four tumors and 14 normal breast samples were also immediately snap frozen in liquid nitrogen and stored at -70°C. Tumors were treated by simple mastectomy or lumpectomy with axillary node sampling. All had axillary node status confirmed histologically. Grading was performed according to the modified Bloom and Richardson method [15]. The characteristics of all patients and tumors are detailed in Table 1.

Follow-up for all patients was conducted every three months for the first 18 months, and 6 monthly until 3 years. In all patients adjuvant radiotherapy was administered to the ipsilateral axilla if lymph nodes had histological evidence of metastasis. Patients with confirmed recurrent disease were treated by endocrine manipulation for soft tissue or skeletal disease or by chemotherapy for visceral disease or failed endocrine therapy. Patients with isolated soft tissue relapse additionally received radiotherapy. Adjuvant treatment is shown in Table 1.

Immunohistochemistry

Two hundred and eleven tumors were stained for JC70 (anti-CD31) (Dako, UK) and cryostat sec-

Table 2. The number of cases positive for CAM expression in control and tumor endothelium and the proportion of vessels compared to PECAM-1 expression

Antigen (n)	PECAM	ICAM-1	VCAM-1	E-sel	P-sel
Control (14)	100% (++)	71% (+/–)	0% (–)	21% (+/–)	64% (+)
Tumor (≤64)	100% (++)	69% (++)	10% (+/–)	*52% (+)	*59% (+)

The percentage of cases showing staining is shown for each marker and the intensity of staining in parentheses is represented using the following qualitative score: ++ all vessels (>75%); + some vessels (25–75%); +/– occasional vessels (<25%); – no staining. * = change in distribution of staining.

tions (8μm) from 12 controls and 64 of the tumors were also stained with 6.5B-5 (ICAM-1; D Haskard, London), 4B2 (VCAM; R&D Systems, Oxford U.K.), 1.2B-6 (E-selectin; D Haskard, London), and 11.8K (P-selectin; HK Nieuwenhuis, Amsterdam). Immunohistochemistry was performed using the alkaline phosphatase anti-alkaline phosphatase (Dako, UK) and streptavidin-biotin-peroxidase (Dako Duet Kit, Dako, UK) techniques. Omission of the primary antibody was used as a negative control.

Estrogen receptor and epidermal growth factor receptor

Estrogen receptor (ER) content was determined using an ELISA technique (Abbott Laboratories, USA). Tumors were considered positive when ER levels exceeded 10 fmol/mg cytosolic protein. Epidermal growth factor receptor (EGFR) was measured by ligand binding of ^{125}I-EGF to tumor membranes. Concentrations greater than 20 fmol/mg membrane protein were considered positive as previously reported [9].

Assessment of tumor vascularity

Chalkley counts were determined without knowledge of patient outcome. The three most vascular areas where the highest number of discreet microvessels stained were chosen by two observers over a conference microscope. Microvessels were defined as any immunoreactive endothelial cell(s) separate from adjacent microvessels. Vessels within the sclerotic body of the tumor were not included. These maximal areas of neovascularization were identified by scanning at low power (x40 and x100). Vessels were then estimated by both observers using a 25 point Chalkley eyepiece graticule [16] at X250 magnification (the graticule covered an area of 0.155 mm^2 at this magnification). The graticule was rotated in the eyepiece to where the maximum number of graticule dots overlay immunohistochemically identified vessels or their lumens. Chalkley counts for individual tumors were then produced using the mean of the three graticule counts.

Statistics

The relationships between the different parameters were examined by converting the continuous variables into categorical variables using cut points of the continuous variables corresponding to the 33% and 67% tertiles, thus dividing data into three equally sized groups. Chi squared tests were then performed for the analysis of these categories. For Chalkley count, stratifying by the cut-offs outlined above, the log rank test and a multivariate Cox proportional hazard model were used to investigate statistical differences in overall survival. The statistical analysis was performed using the Stata package release 3.1 (Stata Corporation, 702 University Drive East, College Station, Texas 77840).

Figure 1. E-selectin (A) and P-selectin (B) staining in an invasive breast carcinoma demonstrating intense endothelial immunoreactivity (arrows), most prominent at the tumor periphery. There is also focal tumor cell positivity for P-selectin.

Results

Expression of CAMs in normal breast tissue

In normal breast tissue we observed strong constitutive endothelial cell expression of PECAM and P-selectin, compared to weaker and more focal expression of ICAM-1 and only very occasional single vessel positivity for E-selectin (Table 2). PECAM and P-selectin were distributed evenly throughout the samples in all vessel sizes, whilst ICAM-1 and E-selectin were generally confined to small caliber vessels. No VCAM-1 immunoreactivity was present in the endothelium of normal breast. In 5/14 controls ICAM-1 stained in the myoepithelial cells of ducts and acini, but no other elements of the breast expressed immunoreactivity for any of the other markers.

Expression of CAMs in breast tumor endothelium

There was a marked increase in the expression of several CAMs in the tumors (Table 2). Endothelial reactivity was intense and widespread with PECAM. In contrast to the control tissues, a significant proportion of the tumor endothelial cells also showed positive staining for both selectins. VCAM expression although upregulated was focal and weak, whilst PECAM, ICAM-1, and E-selectin demonstrated more widespread and intense immunoreactivity. Spatially, whereas PECAM immunoreactivity was consistently observed in endothelial cells throughout the tumor, expression of E- and P-selectins was more prominent at the tumor periphery.

Expression of CAMs in breast tumor cells

ICAM-1 and E- and P-selectins stained the cellular neoplastic element of the tumors in 22/64 (34%), 4/61 (7%), and 20/54 (37%) cases, respectively (Figure 1). PECAM reactivity was seen in one case, 1/64 (1.6%). All tumor cells were negative for VCAM.

Relationship of tumor vascularity to tumor characteristics and patient survival

Tumor vascularity ranged from 2.67-9.33 Chalkley counts per x250 magnification (median 5.67). The 33% and 67% tertile bands were at 5 and 7. There was a significant correlation between Chalkley count and lymph node status ($p=0.05$), but no significant correlation between Chalkley count and patient age ($p=0.43$), histology ($p=0.1$), grade ($p=0.22$), tumor size ($p=0.25$), ER status

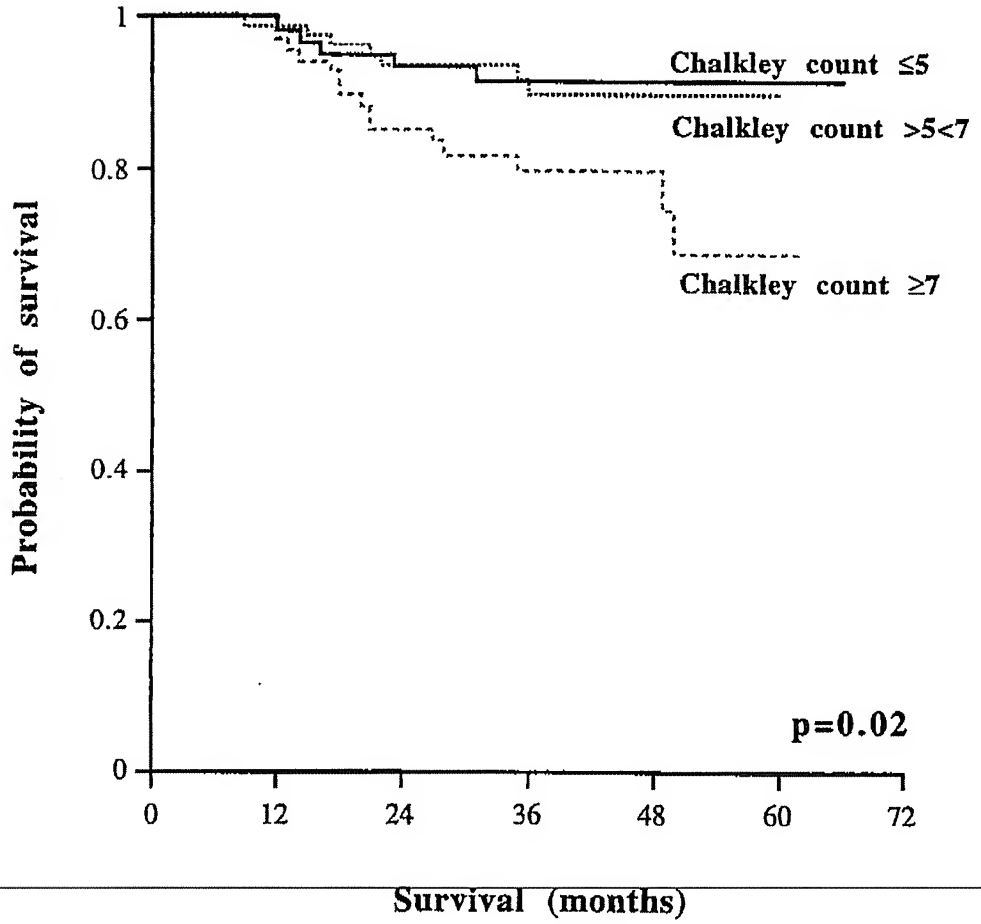

Figure 2. Survival curves stratified by Chalkley count tertiles.

(p=0.86), or EGFR status (p=0.82).

A univariate and multivariate analysis of survival in these patients showed that Chalkley count was associated with a significant reduction in overall survival (p=0.02 and p=0.05, respectively) (Figure 2) (Table 3).

Discussion

We have extended our previous analysis of breast tumor vascularity using Chalkley counting to 211 cases, and examined overall survival in these patients. In a subset of 64 of these tumors we have also used immunohistochemistry to examine the expression of CAMs on the tumor endothelium and cells.

We have demonstrated, stratifying by thirds, that Chalkley counts give independent prognostic information on overall patient survival. Indeed, in the multivariate model Chalkley counting was second in significance only to node status in predicting death. Previously we had used the median

Table 3. Results of a multivariate Cox proportional hazard analysis for overall survival of 211 patients

Prognostic indicator	Hazard ratio	95% CI	p-value
Age	1.03	0.99,1.08	0.17
Tumor size	1.14	0.8,1.6	0.46
ER	0.3	0.99,1.0	0.44
EGFR	1.0	1.0,1.02	0.17
Lymph nodes	1.2	1.1,1.3	0.0001
Chalkley count	1.7	0.9,2.9	0.05

CI-confidence interval

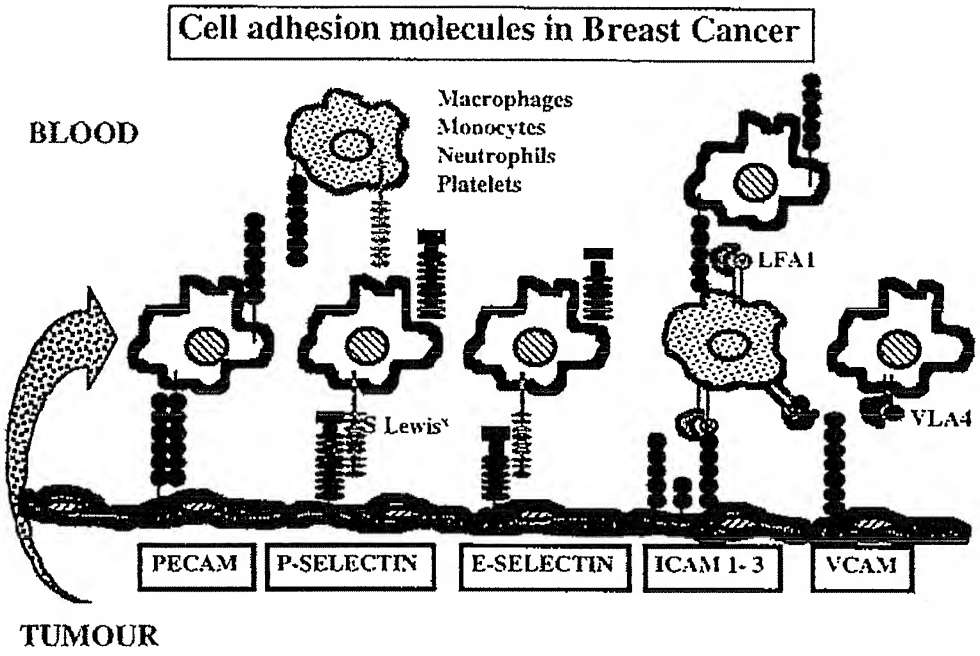

Figure 3. Tumor cells secrete cytokines activating tumor associated endothelium. Tumor cells migrate (arrow) into the vascular lumen and assemble by interaction with CAMs prior to metastasis. Macrophages adhere to tumor clusters and endothelium and can migrate into the tumor bed and release further cytokines and angiogenic factors.

value Chalkley count to stratify patients [9]. In this paper tertiles were selected because, as with the median, this categorization avoids strong assumptions about the relationship between tumor vascularity and other variables, but also reduces loss of data and identifies a similar proportion of patients who have a poor outcome when identified by cut-point analysis [5-7].

In accordance with our own and other studies, and in addition to the confirmation of angiogenic counts giving independent prognostic information, there was a significant correlation between tumor vascularity and lymph node status [3-5]. However, we observed no similar correlation between tumor vascularity and other prognostic markers such as tumor size, grade, and estrogen receptor status. None of these other markers added further prognostic information to lymph node status plus angiogenic count, as shown in Table 3.

Using immunohistochemistry we have observed the expression of the constitutive markers PECAM-1 and P-selectin on breast tumor endothelium in a similar pattern to controls. However there was up-regulation of the intensity of ICAM-1 staining and the intensity and pattern of E-selectin immunoreactivity on tumor endothelium compared to normal breast tissue. Furthermore, in contrast to the pan-endothelial staining of PECAM, there was preferential endothelial cell expression of the selectins at the tumor periphery.

This up-regulation and change in pattern of selectin expression may have a role in tumor-endothelial cell interaction (Figure 3). Acquisition of selectin ligands by cells at the tumor periphery may help assembly of clusters of tumor cells prior to escape into the circulation. They may also promote transmigration of macrophages from the circulation into tumor tissue by adhesion to activated endothelium. This may facilitate tumor-macrophage interactions benefiting tumor growth, and enhance angiogenesis through further release by macrophages of cytokines and angiogenic factors.

Furthermore the expression of selectins on endothelium at the tumor periphery, where angiogenesis is most active [17], may play a direct role

in tumor angiogenesis. In vitro evidence suggests that selectins are involved in capillary morphogenesis and that ligand binding to selectins on endothelial cells alters their morphology and function [18,19]. In view of these findings we are currently examining the expression of selectin ligands in breast tumors and correlating their expression at sites of metastasis such as lymph nodes.

Since no significant up-regulation of VCAM-1 on breast tumor endothelium was observed, this CAM is unlikely to play a major role in determining metastasis in breast tumors, unlike other tumor types such as melanomas [14, 20-22]. This difference in the patterns of CAM expression between different tumor types may provide a molecular basis for inter-tumor variation in the specific site of metastasis, and implies that CAM expression is controlled by growth factors secreted by the tumor rather being a non-specific response to host factors.

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EXHIBIT D

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The claudin gene family: expression in normal and neoplastic tissues

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Abstract

Background: The claudin (*CLDN*) genes encode a family of proteins important in tight junction formation and function. Recently, it has become apparent that *CLDN* gene expression is frequently altered in several human cancers. However, the exact patterns of *CLDN* expression in various cancers is unknown, as only a limited number of *CLDN* genes have been investigated in a few tumors.

Methods: We identified all the human *CLDN* genes from Genbank and we used the large public SAGE database to ascertain the gene expression of all 21 *CLDN* in 266 normal and neoplastic tissues. Using real-time RT-PCR, we also surveyed a subset of 13 *CLDN* genes in 24 normal and 24 neoplastic tissues.

Results: We show that claudins represent a family of highly related proteins, with claudin-16, and -23 being the most different from the others. From *in silico* analysis and RT-PCR data, we find that most claudin genes appear decreased in cancer, while *CLDN3*, *CLDN4*, and *CLDN7* are elevated in several malignancies such as those originating from the pancreas, bladder, thyroid, fallopian tubes, ovary, stomach, colon, breast, uterus, and the prostate. Interestingly, *CLDN5* is highly expressed in vascular endothelial cells, providing a possible target for antiangiogenic therapy. *CLDN18* might represent a biomarker for gastric cancer.

Conclusion: Our study confirms previously known *CLDN* gene expression patterns and identifies new ones, which may have applications in the detection, prognosis and therapy of several human cancers. In particular we identify several malignancies that express *CLDN3* and *CLDN4*. These cancers may represent ideal candidates for a novel therapy being developed based on CPE, a toxin that specifically binds claudin-3 and claudin-4.

Background

The claudin family consists of approximately 23 proteins that are essential for the formation of tight junctions (TJs) in epithelial and endothelial cells [1]. TJs have crucial roles in the control of paracellular transport and in the maintenance of cell polarity. It is thought that various claudin family members can confer different properties to epithelial cell permeability and account for some of the selective variability of different barriers [1]. Indeed, most tissues express multiple claudins, which can interact in both homotypic and heterotypic fashion to form the tight junction strands. The exact combination of claudin proteins within a given tissue is thought to determine the selectivity and strength of the tight junctions. Underscor-

ing the critical roles of claudin proteins are recent observations that germline mutation in these genes can lead to various familial diseases, such as neonatal sclerosing cholangitis (*CLDN1*) [2], nonsyndromic recessive deafness (*CLDN14*) [3], and familial hypomagnesaemia (*CLDN16*)[4].

Recent gene expression profiling analyses have shown that claudin gene expression is frequently altered in various cancers (reviewed in [5,6]). For example, *CLDN3*, and *CLDN4* have been found frequently up-regulated in ovarian, breast, prostate and pancreatic tumors [7-11]. *CLDN7* has been found downregulated in breast and head and neck cancer, but elevated in stomach cancer [12,13]. *CLDN1* is typically downregulated in various cancers, but has also been reported to be elevated. The picture that emerges suggests that claudin expression is altered in several human tumors. Specifically, *CLDN1,3,4,5,7,10,16* have been found altered in various cancers [5]. The overexpression of these proteins in cancer (which typically lose their TJs) is unexpected but may be related to roles that are unrelated to TJ formation [5]. Indeed, recent work suggests that claudins may be involved in survival and invasion of cancer cells [12,14,15].

Regardless of their exact functions in cancer cells, claudin protein expression may have significant clinical relevance [5,6]. For example, claudin-1 expression has been shown to have prognostic value in colon cancer [16], claudin-18 in gastric cancer [17], and claudin-10 in hepatocellular carcinoma [18]. In addition, because claudins are surface proteins, they may represent useful target for various therapeutic strategies. Of particular interest, in the possible use of *Clostridium perfringens* enterotoxin (CPE) as a novel chemotherapeutic compound. CPE is a natural ligand for claudin-3 and -4 proteins, and binding of the toxin to these claudins leads to a rapid cytolysis of the cells [19]. Recent preclinical studies have suggested that CPE may be effective against claudin-3 and -4-expressing malignancies [8,9,11,20].

Unfortunately, the exact patterns of expression of the various claudins in different cancers and normal tissues are not well known. To date, only a few of the claudin proteins have been investigated in a relatively limited number of cancers. In this report, we use the vast amount of data present in the public SAGE database to create a claudin gene expression profile of all the known claudin genes, in a large number of tissues. We then survey a subset of these claudin genes using real-time RT-PCR in a panel of normal and neoplastic tissues. Our study confirms previous claudin gene expression patterns and identifies new ones, which may potentially be of clinical use for various cancers.

Methods

Claudin homology and phylogenetic tree

21 human claudin genes and corresponding proteins sequences were identified and downloaded from GenBank. The ClustalW software (with the Blosum62 matrix) was used to produce a multiple sequence alignment of all these human claudin protein sequences and the Jalview software was then used to visualize the results [21]. A phylogenetic tree of the claudin proteins was produced with ClustalW. The clustalW phylogenetic calculations are based on the neighbor-joining method of Saitou and Nei [22].

In silico analysis of claudin gene expression

Mining of the SAGE Genie database [23] for libraries that expressed *GAPDH* or *ACTB* yielded a total of 266 SAGE libraries with at least some level of expression of these control genes. These libraries were then examined for the expression of all 21 human *CLDN* genes that we identified. SAGE data for both normal and cancerous tissues was exported to an excel spreadsheet, and expression levels converted to tags per 200,000 (the complete dataset is available as additional file 1). The dChip software http://www.dchip.org program was then used to visualize this data, assigning darker shades of red to higher number of tags.

Real-time RT-PCR of claudin family members

A total of 48 cDNA preparations from various normal and neoplastic tissues (24 each) were purchased from Biochains (Hayward, CA). The GeneAmp 7300 Sequence Detection System (PE Applied Biosystems) was used for detecting RT-PCR products in real-time with the SYBR Green I assay, as previously described [24]. The primers for the various *CLDN* genes (*CLDN1,2,3,4,5,7,8,9,10,11,12,16,18*) and the control *GAPDH* were designed to cross intron-exon boundaries to distinguish PCR products generated from genomic versus cDNA template. For *CLDN* genes lacking introns, real-time RT-PCR was performed by the polyA cDNA-specific RT-PCR method [25]. The primer sequences are available online as additional file 2.

Each PCR reaction was optimized to ensure that a single band of the appropriate size was amplified and that no bands corresponding to genomic DNA amplification or primer-dimer pairs were present. The PCR cycling conditions were performed for all samples as follows: 50°C, 2 minutes for AmpErase UNG incubation, 95°C, 10 minutes for AmpliTaq Gold activation, and 40 cycles for the melting (95°C, 15 seconds) and annealing/extension (60°C for 1 minute) steps. PCR reactions for each template were done in duplicate in 96-well plates.

The comparative C_T method (PE Applied Biosystems, Foster City, CA) was used to determine relative quantitation of gene expression for each *CLDN* gene compared to the *GAPDH* control. First, the C_T values from *GAPDH* reactions were averaged for each duplicate. Next, the relative difference between *GAPDH* and each duplicate was calculated as previously described [24]. The final values were then averaged for each duplicate set, and used in the dChip analysis. Clustering of the *CLDN* genes was performed with distances based on 1-rank correlation and the centroid linkage method.

Results

The claudin family of proteins

As a starting point for our analyses, we identified 21 different human claudin proteins in the GenBank database. Alignment of these 21 sequences using ClustalW shows that most of the claudin proteins are extremely similar, especially in the membrane-spanning regions (Figure 1A). Notable exceptions are claudin-16, which contains a 66 aa extension at the N-terminus, claudin-18, which has an extension in the second extracellular loop, and claudin-23 with a longer C-terminal tail. A phylogenetic tree was also generated to better identify similar members of the family. Overall, the tree demonstrated that the claudins constitute highly related family of proteins, with claudin-16, and -23 being the most different from the others (Figure 1B). Claudin-6 and -9 are the most similar, followed by claudin-3 and -4, and claudin-1 and -7.

In silico analysis of CLDN expression in 266 tissues

Because serial analysis of gene expression (SAGE) measures absolute levels of transcripts, cross-comparison of SAGE data is possible across experiments and laboratories [26]. The SAGE Genie database has been developed to allow *in silico* analysis of gene expression and comparison of transcript levels in a large number of normal and diseased tissues [23]. Using the SAGE Genie database, we extracted gene expression data for all 21 human claudins across 266 tissues (Figure 2 and additional file 1). Some of these genes, such as *CLDN1,2,3,4,5,7,11,12*, and *15* are expressed in a large number of different tissues. In contrast, other claudins, such as *CLDN14,16,17,20*, and *22* have much more restricted expression patterns. *CLDN17*, for example, was found expressed in only one SAGE library (normal kidney), at low levels. *CLDN20* was only found in 3 cancer libraries total (a chondrosarcoma, a brain cancer, and a liver tumor). Similarly, *CLDN22* was only found in 2 breast cancer libraries and one brain astrocytoma library. On the other hand, *CLDN3,4*, and *7* were highly expressed in most normal epithelial cells as well as their corresponding neoplasias. Further analysis suggested that these claudins are frequently elevated in cancer. For example, *CLDN3* was elevated in tumors of the lung, prostate, breast, kidney, and ovary compared to their normal counterparts. Similarly, *CLDN4* was elevated in tumors of the lung, breast, stomach, pancreas, and ovary. *CLDN7* was elevated in cancers of the thyroid, lung, stomach, pancreas, liver, kidney, and ovary. Interestingly, *CLDN6* was frequently expressed in embryonic stem cells (ESCs) but generally not in other tissues. *CLDN7* was the only other *CLDN* expressed at significant level in ESCs. *CLDN18* expression seemed to be mostly restricted to the stomach and the lungs. Vascular endothelial cells expressed *CLDN5* at high levels, suggesting a new target for antiangiogenic therapy. Brain had distinctive claudin expression profiles, with *CLDN3,4* and *7*, expressed at low levels but *CLDN2* and *CLDN5* very highly expressed. This pattern was opposite to what we observed in epithelial cells. *CLDN12* was the most widely expressed gene and appeared expressed constitutively in most tissues.

Real-time RT-PCR analysis of CLDN expression

In order to validate and extend the *in silico* results obtained with the SAGE Genie database, we performed real-time RT-PCR analysis on a subset of *CLDN* genes (13 genes total) to survey gene expression in several tissues. Gene-specific primers for *CLDN1,2,3,4,5,7,8,9,10,11,12,16,18* were designed and optimized. These *CLDN* genes were chosen because they were expressed at detectable levels in several tissues and represented a wide variety of different expression patterns as suggested by the SAGE database analysis. A total of 24 normal tissues and 24 tumors were surveyed for *CLDN* expression (Figure 3). Using this technique, we find that the various normal tissues express a wide variety of *CLDN* genes (Figure 3A). For example, the kidney expresses high levels of *CLDN10* and *CLDN12*, but also expresses some levels of all the other *CLDN* genes tested (with the exception of *CLDN18*). Clustering of *CDLN* genes showed a tight association of *CLDN3,4,7* in terms of their expression patterns, suggesting a coordinate regulation of these genes. The *CLDN3,4,7* cluster was found expressed at high levels in normal pancreas, salivary gland, kidney, adrenal gland, small intestine, colon, and thyroid. Examination of the tumor samples revealed that the diversity of *CLDN* expression was decreased in these samples (Figure 3B). Except for the *CLDN3,4,7* cluster, which was also present and often elevated in tumors, the other *CLDN*s appeared to be expressed at relatively low levels. *CLDN3,4,7* were expressed in tumors of the pancreas, bladder, thyroid, fallopian tubes, ovary, stomach, colon, breast, uterus, and prostate. Because of the low number of samples examined, this survey doe not represent an exhaustive analysis of *CLDN* gene expression in various tissues but rather an initial study of tissue specificity. However, the similarity in the gene expression patterns identified through this survey and previously known patterns (for *CLDN3* and *4*, for example) is striking. Additional studies with several

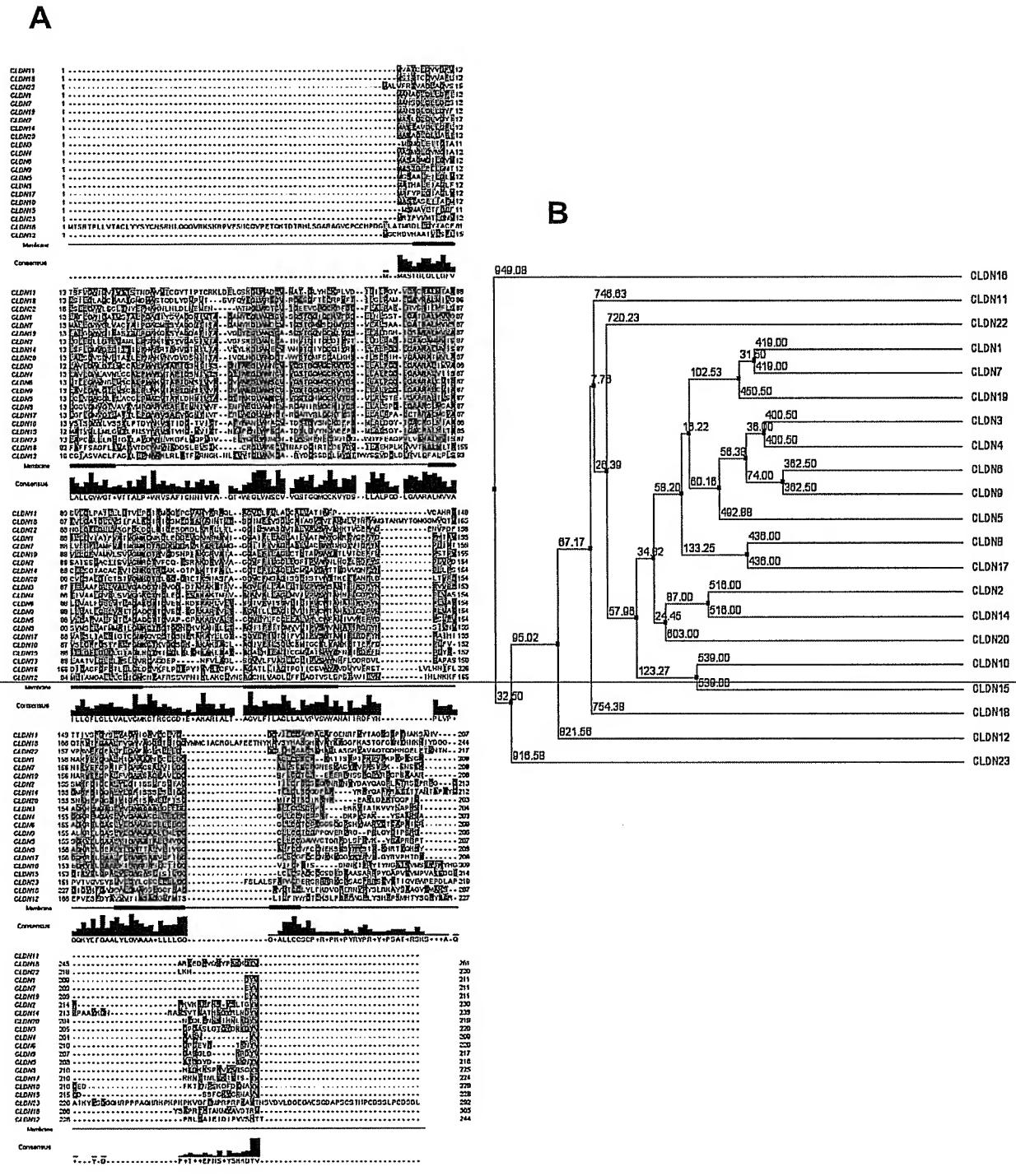

Figure 1

Multiple alignment and family tree of claudin proteins. A. ClustalW was used to generate alignment of all the human claudin protein sequences and the residues were then colored using Jalview [21] according to amino acid conservation among family members. Red bars underneath alignment indicate predicted transmembrane domains. The level of conservation and the consensus are also shown below the alignment. B. Phylogenetic tree of claudin family members. The tree was generated using ClustalW and visualize in Jalview. The numbers on the branches are Blosum62 scores, indicating distances among family members.

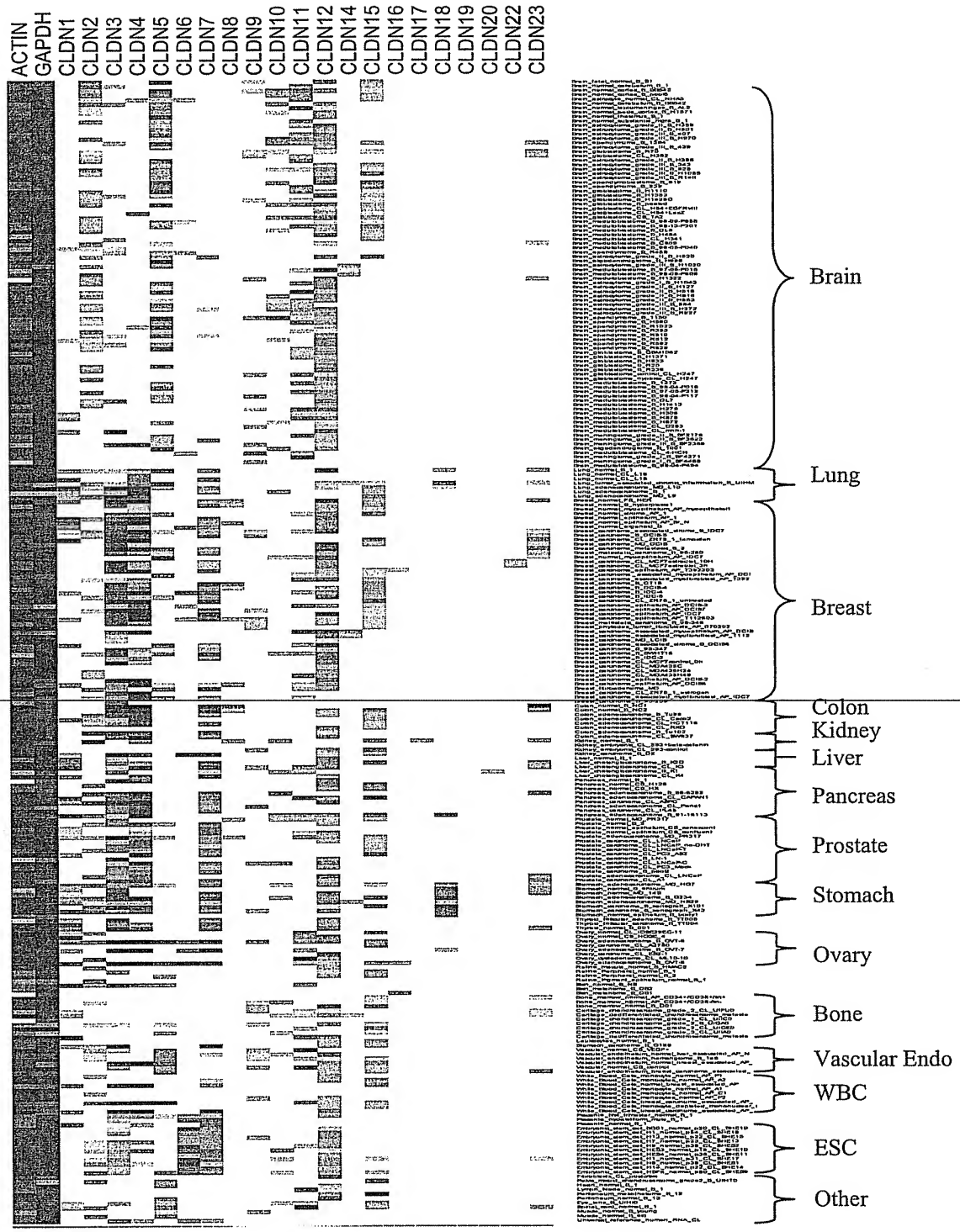

Figure 2

In silico analysis of claudin gene expression in various normal and neoplastic tissues. 266 SAGE libraries were examined for the expression of all 21 *CLDN* genes using SAGE Genie [23]. SAGE data was compiled from both normal and cancerous tissue, and analyzed using dChip software http://www.dchip.org program, which assigned darker shades of red to higher number of tags. GAPDH and ACTN levels were also analyzed as controls. While some *CLDN* genes are ubiquitously expressed, others exhibit highly tissue specific patterns. A detailed spreadsheet of the data is available as supplementary information.

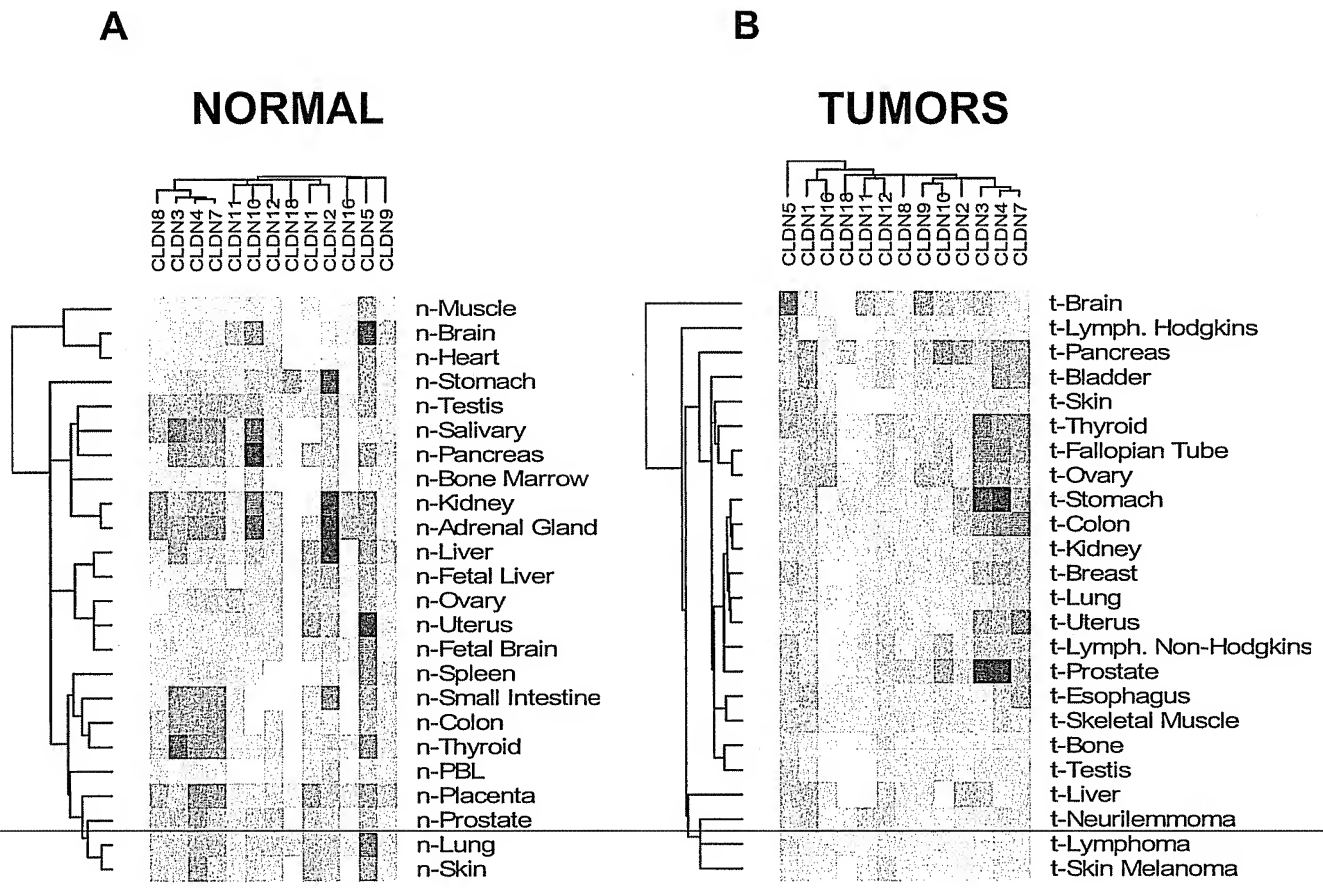

Figure 3
Real-time RT-PCR survey of *CLDN* gene expression in normal and neoplastic tissus. Expression data of 13 selected *CLDN* family members from 24 normal and 24 tumor tissue cDNAs. The dChip software was used to analyze and cluster the data. Clustering of the *CLDN* genes and the tissues were performed with distances based on 1-rank correlation and the centroid linkage method. Darker shades of red indicate higher *CLDN* expression.

tumors, including various subtypes, grades, stages, and other clinical parameters will be necessary.

Discussion

Alterations in the expression levels of tight junction proteins, especially claudins, continue to be reported in several cancers. However, an overall view of claudin gene expression in normal and cancer tissues has been lacking. In this report, we first use the large public SAGE database to investigate claudin expression of the 21 human *CLDN* genes we have identified in GenBank. We find that, while some *CLDN* genes are ubiquitously expressed, the majority of these genes exhibit a very restricted expression pattern. *CLDN14,16,17,19,20,* and *22,* for example, are found in only a few rare libraries. Others such as *CLDN3,4,5,7,11,* and *12* are much more widely expressed. Our analysis allows for the identification of general expression patterns, such as the high expression of *CLDN3,4* and *7* in epithelial tissues, and lower expression in other tissues, such as the brain.

Our data also reveal claudin expression patterns that were not previously known and that may have clinical implications. According to our data, gastric cells (both normal and neoplastic) express high levels of *CLDN18,* while other tissues do not express this gene. Interestingly, a recent study shows that claudin-18 is highly expressed in normal gastric cells and that this high expression is retained in approximately half the gastric tumors [17]. Because of its highly restricted pattern, claudin-18 may therefore represent a useful target for therapy of gastric cancer, especially in those tumors that maintain high levels of this gene. Claudin-18 is likely involved in TJ formation in normal gastric cells, while cancer cells, which typically do not form TJ's, may have a more available form of claudin-18. Therefore cancer cells may be more sensi-

tive to therapy involving the targeting of this molecule. We also find that *CLDN5* is not generally expressed in epithelial tissues but is expressed at high levels in all vascular endothelial cell libraries analyzed. Although also expressed in the brain, *CLDN5* may represent a target for antiangiogenic therapy, especially if using compounds that cannot cross the blood-brain barrier.

Our RT-PCR experiments provide a more quantitative look at claudin gene expression in several normal and neoplastic tissues. It is important to note that these RT-PCR investigations do not represent an exhaustive study of *CLDN* gene expression, but rather a survey of expression in a large number of different human tissues. Follow-up studies on multiple samples for these different malignancies will be necessary to clearly establish the extent and levels of expression of claudins in these tissues. However, it is important to note that the patterns of gene expression obtained by real-time RT-PCR (Figure 3) closely mirrors the *in silico* findings using the SAGE Genie database (Figure 2). For example, the *CLDN3* and *CLDN4* expression patterns are consistent between the two analyses (as described above). In addition, we also observe high correspondence in the two approaches when examining *CLDN5* expression, which appears to be especially high in normal brain and brain cancer. Interestingly, when clustering our RT-PCR data for gene expression patterns, we find *CLDN3,4,7* are very similar in their expression, suggesting coordinate regulation. The fact that the *CLDN3,4,7* cluster is present in both normal and tumors suggests that the mechanisms that lead to the coordinated expression of claudins in normal cells is conserved in tumor cells, although it may be inappropriately activated in cancer. It will certainly be interesting to elucidate the mechanisms that lead to the inappropriate activation of these genes.

Our *in silico* and RT-PCR results are consistent with numerous previous reports showing that *CLDN3* and *CLDN4* are overexpressed in breast [11], ovarian [7], and prostate tumors [9]. In addition, our data showing overexpression of *CLDN4* in pancreatic cancer is also in agreement with previous reports [8,27]. The finding of expression of these claudins in other tumors, such as bladder, thyroid, fallopian tubes, stomach, colon, and uterus, is novel and warrants further investigation. CPE-based therapy, which specifically targets cells expressing claudin-3 or claudin-4 [8,9,11,20], may be worth exploring in these malignancies as well. The fact that *CLDN3*, and *CLDN4* are expressed in several normal tissues (Figure 3A) certainly suggests that systemic administration of CPE may have significant toxic effects. However, the therapeutic index of this compound will depend on the level of up-regulation in the various tumors under study and the mode of administration. In ovarian cancer, for example, where both *CLDN3* and *CLDN4* are highly up regulated and where intraperitoneal therapy is possible, CPE treatment is certainly an interesting possibility.

In this report we study the expression of the *CLDN* genes at the mRNA level, but it will obviously be essential to validate these findings at the protein level when all the antibodies are available, as posttranslational mechanisms have been shown to regulate claudin protein levels and localization [5]. In addition, it will be important to investigate the various claudins studied here for their potential clinical use in cancer therapy and diagnosis. With over 20 known members, many of which, as we show in this report, exhibit high tissue-specific expression and deregulation in various cancers, the claudin family of membrane proteins may represent ideal targets for cancer diagnosis and therapy.

Conclusion

Systematic analysis of CLDN gene expression using *in silico* and RT-PCR approaches demonstrate a wide range of expression patterns among the various claudins in human cancer. *CLDN3*, *CLDN4*, and *CLDN7* are elevated in several malignancies such as those originating from the pancreas, bladder, thyroid, fallopian tubes, ovary, stomach, colon, breast, uterus, and the prostate. These cancers are thus ideal candidates for a for a novel therapy being developed based on CPE, a toxin that specifically binds claudin-3 and claudin-4. *CLDN18* is specifically expressed in gastric cells and may represent a marker for gastric tumors. *CLDN5* is highly expressed in vascular endothelial cells, providing a possible target for antiangiogenic therapy. Overall, a better knowledge of claudin expression in normal and neoplastic tissues may provide new opportunities for the detection, prognosis and therapy of several human cancers.

Abbreviations

TJ: tight junction; CPE, *Clostridium perfringens* enterotoxin; RT-PCR, reverse-transcriptase PCR; SAGE: serial analysis of gene expression.

Competing interests

The author(s) declare that they have no competing interests.

Authors' contributions

KJH: Performed RT-PCR experiments, multiple alignments, and drafted a first draft of the manuscript; RA: Designed and tested some of the primers for the RT-PCR experiments; PJM: Designed and coordinated the study, interpreted the data, and drafted the manuscript.

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EXHIBIT E

SEQ ID 408 vs CLDN5

Alignment Report of 'SEQ ID 408 vs CLDN5.meg' - ClustalV (PAM250) : Tuesday, January 16, 2007 5:02 PM

```
     M G S A A L E I L G L V L C L V G W G G L I L A C G L P M W  Majority
     ----------------+-----------------+-----------------+-
                     10                20                30
     ----------------+-----------------+-----------------+-
   1 M G S A A L E I L G L V L C L V G W G G L I L A C G L P M W  SEQ ID NO 408
   1 M G S A A L E I L G L V L C L V G W G G L I L A C G L P M W  NP_003268 (CLDN5)

     Q V T A F L D H N I V T A Q T T W K G L W M S C V V Q S T G  Majority
     ----------------+-----------------+-----------------+-
                     40                50                60
     ----------------+-----------------+-----------------+-
  31 Q V T A F L D H N I V T A Q T T W K G L W M S C V V Q S T G  SEQ ID NO 408
  31 Q V T A F L D H N I V T A Q T T W K G L W M S C V V Q S T G  NP_003268 (CLDN5)

     H M Q C K V Y D S V L A L S T E V Q A A R A L T V S A V L L  Majority
     ----------------+-----------------+-----------------+-
                     70                80                90
     ----------------+-----------------+-----------------+-
  61 H M Q C K V Y D S V L A L S T E V Q A A R A L T V S A V L L  SEQ ID NO 408
  61 H M Q C K V Y D S V L A L S T E V Q A A R A L T V S A V L L  NP_003268 (CLDN5)

     A F V A L F V T L A G A Q C T T C V A P G P A K A R V A L T  Majority
     ----------------+-----------------+-----------------+-
                     100               110               120
     ----------------+-----------------+-----------------+-
  91 A F V A L F V T L A G A Q C T T C V A P G P A K A R V A L T  SEQ ID NO 408
  91 A F V A L F V T L A G A Q C T T C V A P G P A K A R V A L T  NP_003268 (CLDN5)

     G G V L Y L F C G L L A L V P L C W F A N I V V R E F Y D P  Majority
     ----------------+-----------------+-----------------+-
                     130               140               150
     ----------------+-----------------+-----------------+-
 121 G G V L Y L F C G L L A L V P L C W F A N I V V R E F Y D P  SEQ ID NO 408
 121 G G V L Y L F C G L L A L V P L C W F A N I V V R E F Y D P  NP_003268 (CLDN5)

     S V P V S Q K Y E L G A A L Y I G W A A T A L L M V G G C L  Majority
     ----------------+-----------------+-----------------+-
                     160               170               180
     ----------------+-----------------+-----------------+-
 151 S V P V S Q K Y E L G A A L Y I G W A A T A L L M V G G C L  SEQ ID NO 408
 151 S V P V S Q K Y E L G A A L Y I G W A A T A L L M V G G C L  NP_003268 (CLDN5)

     L C C G A W V C T G R P D L S F P V K Y S A P R R P T A T G  Majority
     ----------------+-----------------+-----------------+-
                     190               200               210
     ----------------+-----------------+-----------------+-
 181 L C C G A W V C T G R P D L S F P V K Y S A P R R P T A T G  SEQ ID NO 408
 181 L C C G A W V C T G R P D L S F P V K Y S A P R R P T A T G  NP_003268 (CLDN5)

     D Y D K K N Y V                                            Majority
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     ---------------
 211 D Y D K K N Y V                                            SEQ ID NO 408
 211 D Y D K K N Y V                                            NP_003268 (CLDN5)
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EXHIBIT F

ATCC

10801 University Blvd • Manassas, VA 20110-2209 • Telephone: 703-365-2700 • FAX: 703-

BUDAPEST TREATY ON THE INTERNATIONAL RECOGNITION OF
THE DEPOSIT OF MICROORGANISMS FOR THE PURPOSES OF PATENT PROCEDURE

INTERNATIONAL FORM

RECEIPT IN THE CASE OF AN ORIGINAL DEPOSIT ISSUED PURSUANT TO RULE 7.3
AND VIABILITY STATEMENT ISSUED PURSUANT TO RULE 10.2

To: (Name and Address of Depositor or Attorney)

Human Genome Sciences, Inc.
Attn: A. Anders Brookes, Patent Attorney
9410 Key West Avenue
Rockville, MD 20850

Deposited on Behalf of: Human Genome Sciences, Inc.

Identification Reference by Depositor:	**ATCC Designation**
DNA Plasmid PS-092 (Ref. Docket PS-092)	203071

The deposits were accompanied by: ___ a scientific description _ a proposed taxonomic description indicated above. The deposits were received July 27, 1998 by this International Depository Authority and have been accepted.

AT YOUR REQUEST: X We will inform you of requests for the strains for 30 years.

The strains will be made available if a patent office signatory to the Budapest Treaty certifies one's right to receive, or if a U.S. Patent is issued citing the strains, and ATCC is instructed by the United States Patent & Trademark Office or the depositor to release said strains.

If the cultures should die or be destroyed during the effective term of the deposit, it shall be your responsibility to replace them with living cultures of the same.

The strains will be maintained for a period of at least 30 years from date of deposit, or five years after the most recent request for a sample, whichever is longer. The United States and many other countries are signatory to the Budapest Treaty.

The viability of the cultures cited above was tested August 5, 1998. On that date, the cultures were viable.

International Depository Authority: American Type Culture Collection, Manassas, VA 20110-2209 USA.

Signature of person having authority to represent ATCC:

Barbara M. Hailey

Barbara M. Hailey, Administrator, Patent Depository

Date: August 7, 1998